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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. §1.53(b)(2)

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TITLE OF THE INVENTION (280 characters max)

A COMPOUND IDENTIFICATION METHOD

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Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.

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Title

A COMPOUND IDENTIFICATION METHOD

Various patent and non-patent references cited in the present application are hereby incorporated by reference in their entirety.

Technical Field of the Invention

The present invention relates to a method for obtaining the identity of one or more display molecules capable of associating with a molecular target. The display molecules initially form part of a library and the present invention devises a method for identifying such display molecules that possess certain properties relative to a target.

Background

Methods for obtaining information about a display molecule that possesses a binding characteristic towards a molecular target are used in the phage display area and are generally known as panning. A typical panning protocol includes a library of phages displaying certain specific polypeptides and a target. When the library and the target are mixed, polypeptides that have an ability to bind to the target will form an association complex. Polypeptides that do not bind are washed away.

In theory, it should be possible to rank the members of the library in accordance with their binding affinity in an elution step. Thus, the best binders could easily be identified following a single mixing step. However, practical experiments show that libraries of many members cannot properly be partitioned for good and bad binders and a second contacting with the target is necessary. Generally, libraries of the size 10^8 to 10^{12} are used to increase the chance of finding a successfully binding polypeptide. Therefore, several rounds are generally required. To obtain a sufficient quantity of phages for performing a second contacting with a molecular target, the phages harbouring the binding polypeptides are amplified using the genetic material (DNA or

RNA) of the eluted binding phages. It is usual to perform 3 to 12 rounds of contact between the increasingly enriched libraries and the target before a minor group of binding peptides can be identified.

Systems that resemble the phage panning have been evolved by the present applicant (WO 02/103008 A2) however with the possibility of displaying other molecules than polypeptides. Other systems using the same principles as the phage panning include WO 98/31700, WO 93/03172, and WO 00/23458.

For some systems, e.g. as disclosed in EP 643 778 B1 and WO 93/06121 A1, it is not immediately possible to perform amplification of the complexes after elution of binding display molecules associated with an identifying nucleic acid.

The present invention aims at providing a method that only requires a single initial contact between a target and a library of display molecules associated with an identifying nucleic acid even for large libraries. Thus, reiterated amplification of display molecules associated with an identifying or coding nucleic acid between each round of contact with the target can be avoided. Moreover, encoding methods not previous available for large libraries becomes at the disposal of the person skilled in the art.

Summary of the Invention

The present invention relates to a method for obtaining display molecule(s) having affinity towards a target, comprising the steps of: a) providing a library comprising a plurality of different display molecules, each display molecule being associated with an identifier oligonucleotide, which codes for the identity of said display molecule, b) contacting the library with a target to allow for an interaction between the display molecules of the library with the target, c) partitioning a fraction enriched with identifier oligonucleotides of display molecules interacting with the target, d) subjecting the fraction to denaturing conditions and subsequently to conditions at which homo-duplexes renatu-

rate, e) recovering the homo-duplexes, and f) deducing from the homo-duplexes the identity of the display molecule(s) interacting with the target.

An ideal initial library comprises an equal amount of each display molecule associated with the identifier oligonucleotide. The display molecule associated with the identifier oligonucleotide is also for short termed "complex" in the following. It is estimated that a standard vial can comprise around 10^{14} complexes. Therefore a library of 10^8 complexes ideally comprises 10^6 copies of each complex.

The contacting between the library and the molecular target, and the subsequent partitioning of a fraction of the complexes or just the identifier oligonucleotides thereof, interacting with the target forms an imbalance in the amount of the individual members of the library. If elution is used for the partitioning, complexes binding with high affinity to the target will be eluted in a relatively higher amounts compared to low affinity binding complexes, which will be eluted in a correspondingly minor amount.

The obtained imbalance at the nucleic acid level is used in the subsequent steps. The identifier oligonucleotide parts of the binding complexes are usually, but not necessarily, amplified by PCR or similar to obtain a higher total amount of oligonucleotides while retaining the proportion between the individual oligonucleotides.

The PCR amplification provides a homo-duplex product. A mixture of homo- and hetero-duplexes is appropriately initiated by subjecting the PCR amplification product to a denaturing process to separate the duplexes into single stranded oligonucleotides. Subsequently, the mixture of single stranded oligonucleotides is allowed to renaturate to form the homo-duplexes. The denaturing step is suitably obtained by heating the nucleic acids above the melting temperature of the duplexes and the hybridisation step is suitably conducted by lowering the temperature below the melting temperature of the homo-

duplexes and in some aspects of the invention also below the melting temperature of at least a part of the hetero-duplexes. In the event the renaturing conditions allow the formation of hetero-duplexes, the presence of various different single stranded oligonucleotides some oligonucleotides may find a perfectly matching partner and form a homo-duplex, while other oligonucleotides will hybridise to non-complementary binding partners and form hetero-duplexes. In the event the renaturing conditions favours formation of homo-duplexes, while formation of hetero-duplexes mainly is avoided, e.g. by choosing a temperature below the melting temperature of the homo-duplexes, but above at least the majority of the hetero-duplexes, the renaturing step result predominately in the formation of homo-duplexes. The present invention takes advantage of the fact that the oligonucleotides most abundant easier will find perfect binding partners and form homo-duplexes.

After the denaturing and subsequent renaturing step, the homo-duplexes are recovered. Usually, recovery is conducted by eliminating or reducing the amount of hetero-duplexes and single stranded oligonucleotides. Several methods are available for reducing the amount of hetero-duplexes and single stranded oligonucleotides, including DHPLC as disclosed in US 5,795,976, and enzymatic degradation. In some aspects of the invention enzymatic degradation is preferred due to the availability of enzymes specifically locating one or more mis-match pairing nucleobases.

The recovered pool of homo-duplexes may not fully be depleted for hetero-duplexes and single stranded oligonucleotides and/or the diversity of the pool may still be too high for a meaningful decoding to reveal a display molecule of interest. In an aspect of the invention, the step of subjecting the fraction to denaturing conditions and subsequently to conditions at which homo-duplexes renaturate and the step of recovering the homo-duplexes is therefore repeated one or more times, i.e. the recovered pool of homo-duplexes is, optionally after nucleic acid amplification, subjected to denaturing conditions to form single stranded oligonucleotides and subsequent to hybridisation con-

ditions to allow for the formation of a new pool of homo-duplexes; and the homo-duplexes is recovered by a suitable method as disclosed elsewhere herein. Between each repetition, a part of the homo-duplexes may be sequenced to establish whether a further repetition is necessary to identify a display molecule of interest.

The identity of the display molecules that possessed the ability of binding to the molecular target is finally revealed by some of the decoding the homo-duplexes, using conventional methods. When a sequence occurs more frequent than others this is normally an indication of the fact that a display molecule having the desired characteristics has been identified.

Detailed Description of the Invention

The target may be of a biological origin or may be synthetic molecular target. Typically, the molecular target stems from an organism selected from human and animals, especially vertebras. However, in other embodiments the target may originate from a plant. In the quest for a compound with therapeutical effect on the human or animal body, the target is usually expected to have an importance in a therapeutically theory that combats a certain disease. In the quest for discovering compounds with plant protective effect, the target is usually expected to originate from an organism that harms the crop or a competing undesired plant. The organism may be a fungus when a compound with fungicide effect is searched for or an insect when a compound having insecticide effect is desired. Optionally, a protein target stemming from a biological origin may be derivatised by altering, adding, or deleting one or more amino acids.

The target may be a protein, a small molecular hormone, a lipid, a polysaccharide, a whole cell, a nucleic acid, a metabolite, a heme group, etc. In a preferred aspect the target is a protein. The protein may serve the function in the organism of being an enzyme, a hormone, a structural element, a regulatory protein, a membrane channel or pump, a part of a signal transducing

cascade, an antibody, etc. Suitable target enzymes include kinases, phosphatates, and proteases. The protein may occur as an independent entity or may be dimers, trimers, tetramers, or polymers and the protein may comprise a prosthetic group. Furthermore, the molecular target may be a soluble or insoluble agglomerate of one or more proteins and one or more substituents occurring in the body or artificial components. In another preferred embodiment, the molecular target is a nucleic acid, such as DNA or RNA aptamer or ribozyme.

The target may be immobilized to a solid support. The solid support can be a bead or the surfaces of a well. The target immobilized on the solid support may also form a stable or quasi-stable dispersion in the media. In a certain embodiment, the target is in solution and all the interaction occur in the solution too. The absence of an immobilization step generally reduces the background noise because there is no background surface to associate to. Thus the result of the assay may be more sensitive. In solution, the only background noise imaginable is when the oligonucleotides or display molecules of the library of complexes binds unspecific to the target molecules. The absence of an immobilization step generally necessitates a subsequent recovery step, e.g. by chromatography.

In certain aspects of the invention, it is preferred to immobilize the target on a solid support. The solid support may be beads of a column or the surface of a container. The immobilisation of the molecular target may ease the removal of the non-binding complexes by washing or similar means. In a certain embodiment, a cleavable linkage between the molecular target and the solid support is present. The cleavable linker is preferably selectively cleavable, that is, the linkage can be cleaved without cleaving other linkages in the target or the complexes. The cleavage of the linkage between the molecular target and the solid support may reduce the contribution from the background, such as complexes associated with the surface of the solid support and not binding to the molecular target.

The target may be obtained in any suitable way. A variety of targets are commercially available, either as purified protein or as the corresponding cDNA. Other protein or peptide targets may be isolated from tissues or mRNA (or the corresponding cDNA) may be extracted from a tissue. Smaller peptides may be synthesised chemically using the standard solid-phase Fmoc peptide synthesis. When nucleic acids are used or included in the molecular target, it may be synthesised using the standard amedite synthesis method or by using the natural machinery.

It may be an advantage to have all or at least a part of the identifier oligonucleotides on a double stranded form during the contacting with the molecular target, as certain nucleic acids may perform a binding interaction or a catalytical action on the components present during the contacting step. Thus, in one embodiment of the invention, the identifier oligonucleotide partly or fully is hybridised to a complementing oligonucleotide.

The identifier oligonucleotide comprises the information necessary for decoding the identity of the display molecule. The identifier oligonucleotide may be analysed directly in some instances to reveal the identity of the display molecules that have performed an interaction with the target. The informative part can be decoded in a standard sequencing machine. In general however, it is preferred to include the informative part of the coupled product in to a suitable vector and transfer the vector to a host organism. The host organisms may then be cultivated on a suitable substrate and allowed to form colonies. Samples from the colonies may be used for sequencing in a sequencing machine. Also, the identification may be conducted using any sequencing method known in the art, including QPCR, microarrays, etc.

Display molecule and identifier oligonucleotide association

The display molecule associated with the identifier oligonucleotide is sometimes herein referred to as a bifunctional complex to indicate that a physical

connection between the display molecule and the identifier oligonucleotide normally is present. However, in certain embodiments of the present invention the association between the display molecule and the identifier oligonucleotide may be spatial, i.e. an identifier oligonucleotide specifies the spatial position of a display molecule. The term "bifunctional complex" is intended also to cover the latter embodiment. The identifier oligonucleotide comprises identifying moieties that identify the display molecule. Preferably, the identifier oligonucleotide identifies the molecule uniquely, i.e. in a library of complexes a particular identifier oligonucleotide is capable of distinguishing the molecule it is associated with from the rest of the display molecules.

The display molecule and the identifier oligonucleotide may be attached directly to each other or through a bridging moiety. In one aspect of the invention, the bridging moiety is a selectively cleavable linkage.

The identifier oligonucleotide may comprise one, two or more codons. The codon sequences can be decoded to identify reactants used in the formation of the display molecule. When the identifier oligonucleotide comprises more than one codon, each member of a pool of chemical entities can be identified and the order of codons is informative of the synthesis step each member has been incorporated in.

The sequence of the nucleotides in each codon may have any suitable length. The codon may be a single nucleotide or a plurality of nucleotides. In some aspects of the invention, it is preferred that each codon independently comprises four or more nucleotides, more preferred 4 to 30 nucleotides.

The identifier oligonucleotide will in general have at least two codons arranged in sequence, i.e. next to each other. Two neighbouring codons may be separated by a framing sequence. Depending on the display molecule formed, the identifier oligonucleotide may comprise further codons, such as 3, 4, 5, or more codons. Each of the further codons may be separated by a

suitable framing sequence. Preferably, all or at least a majority of the codons of the identifier oligonucleotide are separated from a neighbouring codon by a framing sequence. The framing sequence may have any suitable number of nucleotides, e.g. 1 to 20. Alternatively, codons on the identifier oligonucleotide may be designed with overlapping sequences.

The framing sequence, if present, may serve various purposes. In one setup of the invention, the framing sequence identifies the position of the codon. Usually, the framing sequence either upstream or downstream of a codon comprises information, which allows determination of the position of the codons. In another setup, the frames have alternating sequences, allowing for addition of building blocks from two pools in the formation of the library. The framing sequence may also or in addition provide for a region of high affinity. The high affinity region may ensure that the hybridisation of the template with an anti-codon will occur in frame. Moreover, the framing sequence may adjust the annealing temperature to a desired level.

A framing sequence with high affinity can be provided by incorporation of one or more nucleobases forming three hydrogen bonds to a cognate nucleobase. Examples of nucleobases having this property are guanine and cytosine. Alternatively, or in addition, the framing sequence may be subjected to backbone modification. Several back bone modifications provides for higher affinity, such as 2'-O-methyl substitution of the ribose moiety, peptide nucleic acids (PNA), and 2'-4' O-methylene cyclisation of the ribose moiety, also referred to as LNA (Locked Nucleic Acid).

The identifier oligonucleotide may comprise one or two flanking regions. The flanking region can encompass a signal group, such as a flourophor or a radioactive group to allow for detection of the presence or absence of a complex or the flanking region may comprise a label that may be detected, such as biotin. When the identifier oligonucleotide comprises a biotin moiety, the identifier oligonucleotide may easily be recovered.

The flanking region(s) can also serve as priming sites for amplification reactions, such as PCR. The identifier oligonucleotide may in certain embodiments comprise an affinity region having the property of being able to hybridise to a building block. The priming sites for PCR amplification may be identical for all the identifier oligonucleotides to allow for a proportional amplification of the individual identifier oligonucleotides at various stages of the present method. Alternatively, the different priming sites with corresponding primers may be used to favour the amplification of certain groups of complexes in the library.

It is to be understood that when the term identifier oligonucleotide is used in the present description and claims, the identifier oligonucleotide may be in the sense or the anti-sense format, i.e. the identifier oligonucleotide can be a sequence of codons, which actually codes for the molecule or can be a sequence complementary thereto. Moreover, the identifier oligonucleotide may be single-stranded or double-stranded, as appropriate.

During the contacting step the identifier oligonucleotide usually is in double stranded form to minimise any interaction relative to the target. However, it may be suitable to establish the identifier oligonucleotide in single stranded form prior to the formation of a mixture of the hetero- and homo-duplexes. Starting with a double stranded identifier oligonucleotide, a single stranded identifier oligonucleotide may easily be prepared by extension of a forward primer annealed at a priming site of the identifier oligonucleotide.

The display molecule part of the complex is generally of a chemical structure expected of having an effect on the target. When the target is of pharmaceutical importance, the molecule is generally a possible drug candidate. The complex may be formed by tagging a library of different possible drug candidates with a tag, e.g. a nucleic acid tag identifying each possible drug candidate. In another embodiment of the invention, the molecule is encoded, i.e.

formed by a variety of reactants, which have reacted with each other and/or a scaffold molecule. Optionally, this reaction product may be post-modified to obtain the final molecule displayed on the complex. The post-modification may involve the cleavage of one or more chemical bonds attaching the encoded molecule to the identifier in order more efficiently to display the encoded molecule. In still another embodiment the display molecule is a polypeptide formed using the natural machinery, such as the methods disclosed in WO 92/02536, WO 91/05058, and US 6,194,550.

A variety or methods for association of an oligonucleotide to a polypeptide display molecule is available for the skilled person in the art. An option involves the association of a display molecule protein with the mRNA responsible for the formation thereof. This method is generally referred to as mRNA display. Optionally, the mRNA may be substituted with the corresponding cDNA. A method for generation such a single or a library of fusions between a protein and the mRNA responsible for the formation thereof is disclosed in WO 98/31700. The corresponding DNA strand may be attached to the protein using the method disclosed in WO 00/32823. The contents of both patent applications being incorporated in their entirety by reference herein. The method of WO 98/31700 includes providing a RNA stand comprising a translation initiation sequence, a start codon operable linked to a protein encoding sequence, and a peptide acceptor at the 3' end and translating the protein encoding sequence to produce a RNA-protein fusion. According to WO 00/32823 a DNA primer is covalently connected to the 3' end of the mRNA strand and extended by reverse transcriptase a to prepare the complementing DNA strand. The original RNA strand may be digested by RNase H. Another suitable method for generating a library is disclosed in WO 01/90414, the content of which is incorporated herein by reference.

In accordance with another option, the identifier oligonucleotide is associated with a polypeptide display molecule using a method generally referred to as ribosome display. Ribosome display is disclosed in WO 93/03172, the con-

tent of which is included herein by reference. A still further option for association is phage display, in which a polypeptide display molecule is presented on the capsule of the phage and the same capsule harbours the RNA or DNA responsible for the formation of the polypeptide.

A further option for associating the display molecule with an identifier oligonucleotide includes the method disclosed in M. Yonezawa *et al*, Nucleic acid research, 2003, vol. 31, No. 19 e118 (included by reference). The method includes the initial provision of an oligonucleotide connected to biotin and compartmentalization thereof together with a transcription and translation system. The oligonucleotide comprises a fusion gene coding for streptavidin and a polypeptide display molecule. After the formation of the fusion protein in each compartment, the streptavidin part of the fusion protein binds to the biotin moiety of the oligonucleotide, thereby associating the display molecule with the oligonucleotide coding for the identity thereof.

In case the display molecule is a nucleic acid, it may be of the aptamer type, i.e. a library of aptamers comprising constant nucleic acid regions flanking a random oligonucleotide part. The random oligonucleotide part serves the dual function of a nucleic acid display molecule and the identifying oligonucleotide.

The formation of a synthetic encoded molecule generally starts by a scaffold, i.e. a chemical unit having one or more reactive groups capable of forming a connection to another reactive group positioned on a chemical entity, thereby generating an addition to the original scaffold. A second chemical entity may react with a reactive group also appearing on the original scaffold or a reactive group incorporated by the first chemical entity. Further chemical entities may be involved in the formation of the final reaction product. The formation of a connection between the chemical entity and the nascent encoded molecule may be mediated by a bridging molecule. As an example, if the nascent encoded molecule and the chemical entity both comprise an

amine group a connection between these can be mediated by a dicarboxylic acid. A display molecule is in general produced in vitro and may be a naturally occurring or an artificial substance. In an aspect of the invention, a display molecule is not produced using the natural translation system in an *in vitro* process. In other aspects of the invention, the display molecule is a polypeptide produced using the natural translation machinery.

The chemical entities that are precursors for structural additions or eliminations of the encoded molecule may be attached to a building block prior to the participation in the formation of the reaction product leading to the final display molecule. Besides the chemical entity, the building block generally comprises an anti-codon. In some embodiments the building blocks also comprise an affinity region providing for affinity towards the nascent complex.

In a certain aspect of the invention, the reactants or chemical entities are suitably mediated to the nascent encoded molecule by a building block, which further comprises an anticodon. The anti-codon serves the function of transferring the genetic information of the building block in conjunction with the transfer of a chemical entity. The transfer of genetic information and chemical entity may occur in any order, however, it is important that a correspondence is maintained in the complex. The chemical entities are preferably reacted without enzymatic interaction in some aspects of the invention. Notably, ribosomes or enzymes having similar activity do preferably not mediate the reaction of the chemical entities. In another aspect of the invention a ribosome is used to translate an mRNA into a protein using a tRNA loaded with a natural or unnatural amino acid. In still another aspect of the invention, enzymes having catalytic activities different from that of ribosomes are used in the formation of the display molecule.

According to certain aspects of the invention the genetic information of the anti-codon is transferred by specific hybridisation to a codon on a nucleic

acid template. Another method for transferring the genetic information of the anti-codon to the nascent complex is to anneal an oligonucleotide complementary to the anti-codon and attach this oligonucleotide to the complex, e.g. by ligation. A still further method involves transferring the genetic information of the anti-codon to the nascent complex by an extension reaction using a polymerase and a mixture of dNTPs.

The chemical entity of the building block may in certain cases be regarded as a precursor for the structural entity eventually incorporated into the encoded molecule. In other cases the chemical entity provides for the eliminations of chemical units of the nascent encoded molecule. Therefore, when it in the present application with claims is stated that a chemical entity is reacted with a nascent encoded molecule it is to be understood that not necessarily all the atoms of the original chemical entity is to be found in the eventually formed encoded molecule. Also, as a consequence of the reactions involved in the connection, the structure of the chemical entity can be changed when it appears on the nascent encoded molecule. Especially, the cleavage resulting in the release of the entity may generate a reactive group, which in a subsequent step can participate in the formation of a connection between a nascent complex and a chemical entity.

The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction, which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The number of reactive groups, which appears on the chemical entity, is suitably one to ten. A building block featuring only one reactive group is used *i.a.* in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. One, two or more reactive groups intended for the formation of connections are typically present on scaffolds. Non-limiting

examples of scaffolds are opiates, steroids, benzodiazepines, hydantoines, and peptidylphosphonates.

The reactive group of the chemical entity may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a chemical reagent or an enzyme. The cleavage results in a transfer of the chemical entity to the nascent encoded molecule or in a transfer of the nascent encoded molecule to the chemical entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage.

In another aspect, the formation of connection between chemical entity and nascent encoded molecule and the cleavage between chemical entity and the remainder of the building block is conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the nascent encoded molecule is a leaving group of the reaction. In some aspects of the invention, it is appropriate to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and cleavage can also be designed such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

The attachment of the chemical entity to the building block, optionally via a suitable spacer can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the backbone. In general, it is preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of the chemical entity, the attachment point is usually at the 7 position of the purines or 7-deaza-purins or at the 5 position of pyrimidines. The nucleotide may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational spaced sampled by the reactive group is optimized for a reaction with the reactive group of the nascent encoded molecule.

The display molecules of the invention may have any chemical structure. In a preferred aspect, the display molecule can be any compound that may be synthesized in a component-by-component fashion. In some aspects the display molecule is a linear or branched polymer. In another aspect the display molecule is a scaffolded molecule. The term "display molecule" also comprises naturally occurring molecules like α-polypeptides etc, however produced *in vitro* usually in the absence of enzymes, like ribosomes. In certain aspects, the display molecule of the library is a non-α-polypeptide.

The display molecule may have any molecular weight. However, in order to be orally available, it is in this case preferred that the display molecule has a molecular weight less than 2000 Daltons, preferably less than 1000 Dalton, and more preferred less than 500 Daltons.

The size of the library may vary considerably pending on the expected result of the inventive method. In some aspects, it may be sufficient that the library comprises two, three, or four different complexes. However, in most events, more than two different complexes are desired to obtain a higher diversity. In some aspects, the library comprises 1,000 or more different complexes; more

preferred 1,000,000 or more different complexes. The upper limit for the size of the library is only restricted by the size of the vessel in which the library is comprised. It may be calculated that a vial may comprise up to 10^{14} different complexes.

Methods for forming libraries of complexes

The complexes comprising an identifier oligonucleotide having two or more codons that codes for reactants that have reacted in the formation of the molecule part of the complex may be formed by a variety of processes. Generally, the preferred methods can be used for the formation of virtually any kind of encode molecule. Suitable examples of processes include prior art methods disclosed in WO 93/20242, WO 93/06121, WO 00/23458, WO 02/074929, and WO 02/103008, the content of which being incorporated herein by reference as well as methods of the present applicant not yet public available, including the methods disclosed in PCT/DK03/00739 filed 30 October 2003, and DK PA 2003 00430 filed 20 March 2003. Any of these methods may be used, and the entire content of the patent applications are included herein by reference.

Below five presently preferred embodiments are described. A first embodiment disclosed in more detail in WO 02/103008 is based on the use of a polymerase to incorporate unnatural nucleotides as building blocks. Initially, a plurality of template oligonucleotides is provided. Subsequently primers are annealed to each of the templates and a polymerase is extending the primer using nucleotide derivatives, which have appended chemical entities. Subsequent to or simultaneously with the incorporation of the nucleotide derivatives, the chemical entities are reacted to form a reaction product. The encoded molecule may be post-modified by cleaving some of the linking moieties to better present the encoded molecule.

Several possible reaction approaches for the chemical entities are apparent. First, the nucleotide derivatives can be incorporated and the chemical entities

subsequently polymerised. In the event the chemical entities each carry two reactive groups, the chemical entities can be attached to adjacent chemical entities by a reaction of these reactive groups. Exemplary of the reactive groups are amine and carboxylic acid, which upon reaction form an amide bond. Adjacent chemical entities can also be linked together using a linking or bridging moiety. Exemplary of this approach is the linking of two chemical entities each bearing an amine group by a bi-carboxylic acid. Yet another approach is the use of a reactive group between a chemical entity and the nucleotide building block, such as an ester or a hoister group. An adjacent building block having a reactive group such as an amine may cleave the interspaced reactive group to obtain a linkage to the chemical entity, e.g. by an amide linking group.

A second embodiment for obtainment of complexes disclosed in WO 02/103008 pertains to the use of hybridisation of building blocks to a template and reaction of chemical entities attached to the building blocks in order to obtain a reaction product. This approach comprises that templates are contacted with a plurality of building blocks, wherein each building block comprises an anti-codon and a chemical entity. The anti-codons are designed such that they recognise a sequence, i.e. a codon, on the template. Subsequent to the annealing of the anti-codon and the codon to each other a reaction of the chemical entity is effected.

The template may be associated with a scaffold. Building blocks bringing chemical entities in may be added sequentially or simultaneously and a reaction of the reactive group of the chemical entity may be effected at any time after the annealing of the building blocks to the template.

A third embodiment for the generation of a complex includes chemical or enzymatic ligation of building blocks when these are lined up on a template. Initially, templates are provided, each having one or more codons. The templates are contacted with building blocks comprising anti-codons linked to

chemical entities. The two or more anti-codons annealed on a template are subsequently ligated to each other and a reaction of the chemical entities is effected to obtain a reaction product. The method is disclosed in more detail in DK PA 2003 00430 filed 20 March 2003.

A fourth embodiment makes use of the extension by a polymerase of an affinity sequence of the nascent complex to transfer the anti-codon of a building block to the nascent complex. The method implies that a nascent complex comprising a scaffold and an affinity region is annealed to a building block comprising a region complementary to the affinity section. Subsequently, the anti-codon region of the building block is transferred to the nascent complex by a polymerase. The transfer of the chemical entity may be transferred prior to, simultaneously with or subsequent to the transfer of the anti-codon. This method is disclosed in detail in PCT/DK03/00739.

A fifths embodiment also disclosed in PCT/DK03/00739 comprises reaction of a reactant with a site reaction site on nascent bifunctional molecule and addition of a nucleic acid tag to the nascent bifunctional molecule using an enzyme, such as a ligase. When a library is formed, usually an array of compartments is used for reaction of reactants and enzymatic addition of tags with the nascent bifunctional molecule.

Thus, the codons are either pre-made into one or more templates before the encoded molecules are generated or the codons are transferred simultaneously with the formation of the encoded molecules.

After or simultaneously with the formation of the reaction product some of the linkers to the template may be cleaved, however, usually at least one linker is maintained to provide for the complex.

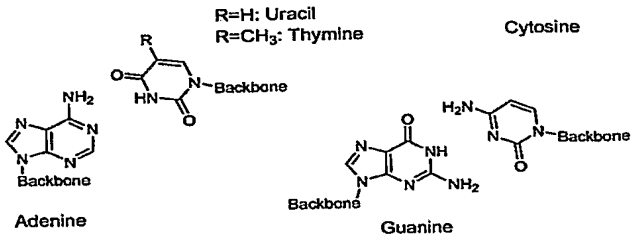
Nucleotides

The nucleotides used in the present invention may be linked together in a sequence of nucleotides, i.e. an oligonucleotide. Each nucleotide monomer is normally composed of two parts, namely a nucleobase moiety, and a backbone. The backbone may in some cases be subdivided into a sugar moiety and an internucleoside linker.

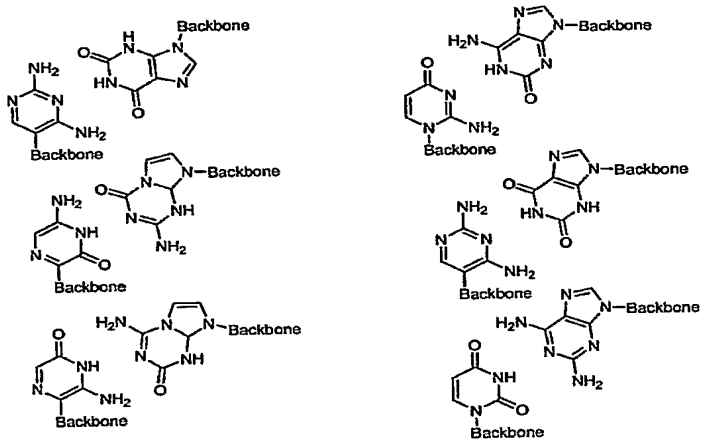
The nucleobase moiety may be selected among naturally occurring nucleobases as well as non-naturally occurring nucleobases. Thus, "nucleobase" includes not only the known purine and pyrimidine hetero-cycles, but also heterocyclic analogues and tautomers thereof. Illustrative examples of nucleobases are adenine, guanine, thymine, cytosine, uracil, purine, xanthine, diaminopurine, 8-oxo-N^6-methyladenine, 7-deazaxanthine, 7-deazaguanine, N^4,N^4-ethanocytosin, N^6,N^6-ethano-2,6-diamino-purine, 5-methylcytosine, 5-(C^3-C^6)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridine, isocytosine, isoguanine, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272. The term "nucleobase" is intended to cover these examples as well as analogues and tautomers thereof. Especially interesting nucleobases are adenine, guanine, thymine, cytosine, 5-methylcytosine, and uracil, which are considered as the naturally occurring nucleobases in relation to therapeutic and diagnostic application in humans.

Examples of suitable specific pairs of nucleobases are shown below:

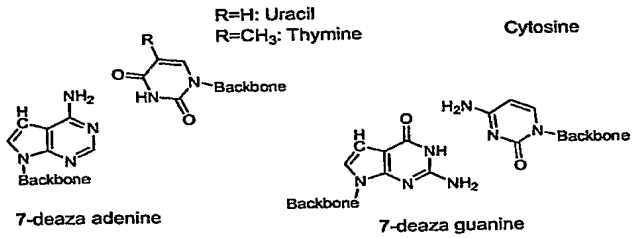
Natural Base Pairs



Synthetic Base Pairs

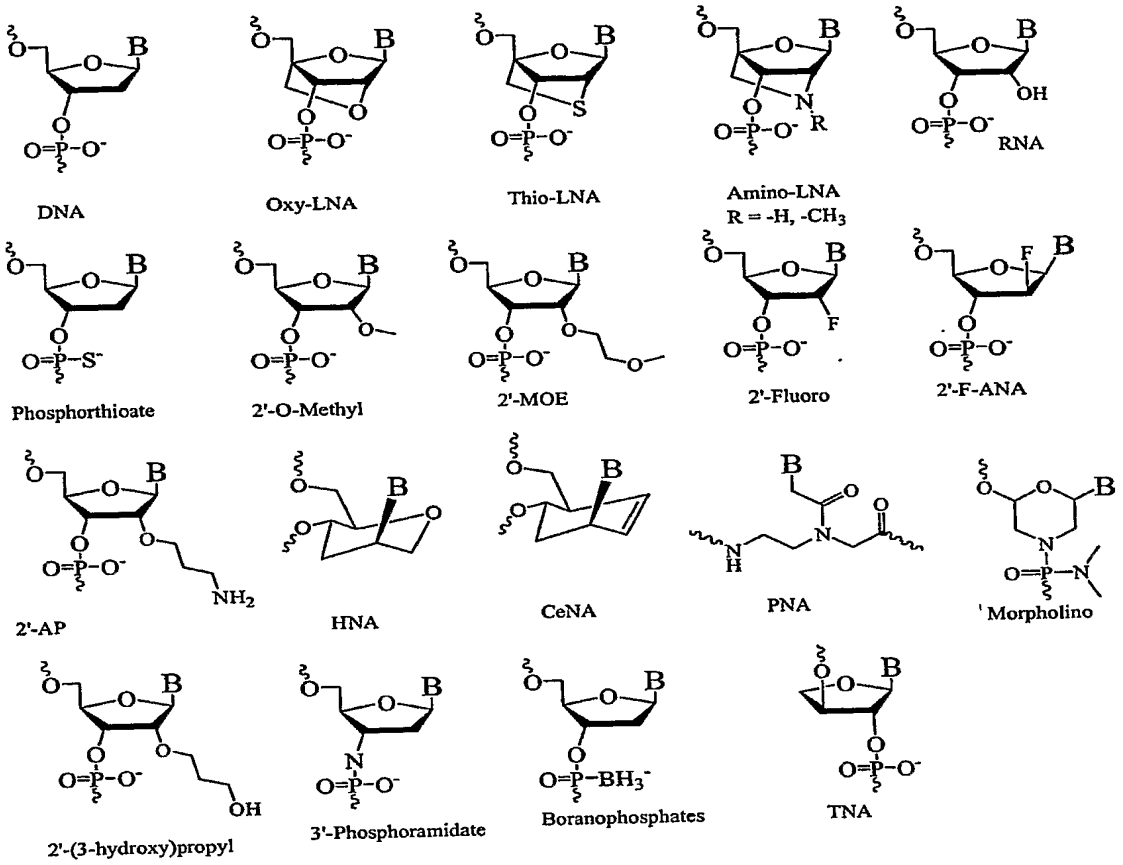


Synthetic purine bases pairring with natural pyrimidines



Suitable examples of backbone units are shown below (B denotes a nucleo-base):

5



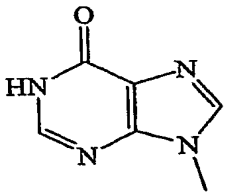
The sugar moiety of the backbone is suitably a pentose but may be the appropriate part of a PNA or a six-member ring. Suitable examples of possible pentoses include ribose, 2'-deoxyribose, 2'-O-methyl-ribose, 2'-flour-ribose, and 2'-4'-O-methylene-ribose (LNA). Suitably the nucleobase is attached to the 1' position of the pentose entity.

An internucleoside linker connects the 3' end of preceding monomer to a 5' end of a succeeding monomer when the sugar moiety of the backbone is a pentose, like ribose or 2-deoxyribose. The internucleoside linkage may be the natural occurring phospodiester linkage or a derivative thereof. Examples of such derivatives include phosphorothioate, methylphosphonate, phosphoramidate, phosphotriester, and phosphodithioate. Furthermore, the

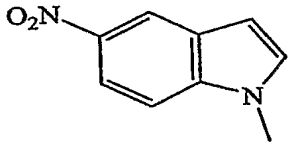
internucleoside linker can be any of a number of non-phosphorous-containing linkers known in the art.

Preferred nucleic acid monomers include naturally occurring nucleosides forming part of the DNA as well as the RNA family connected through phosphodiester linkages. The members of the DNA family include deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. The members of the RNA family include adenosine, guanosine, uridine, cytidine, and inosine. Inosine is a non-specific pairing nucleoside and may be used as universal base because inosine can pair nearly isoenergetically with A, T, and C. Other compounds having the same ability of non-specifically base-pairing with natural nucleobases have been formed. Suitable compounds which may be utilized in the present invention includes among others the compounds depicted below

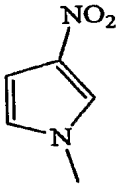
Examples of Universal Bases:



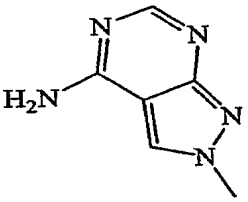
Inosine



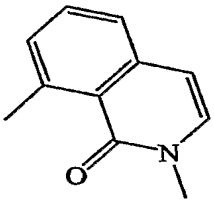
5-Nitroindole



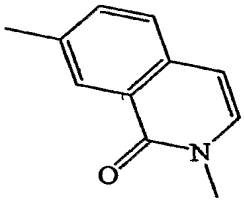
3-Nitropyrrole



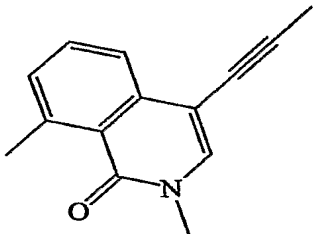
N^8-8aza-7deazaadenine



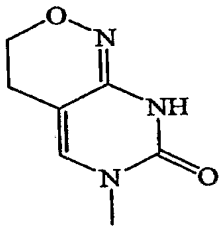
MICS



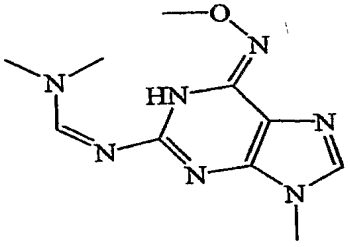
5MICS



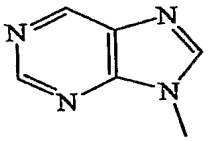
PIM



dP



dK



Nebularine

Building block

The chemical entities or reactants that are precursors for structural additions or eliminations of the encoded molecule may be attached to a building block prior to the participation in the formation of the reaction product leading to the final encoded molecule. Besides the chemical entity, the building block generally comprises an anti-codon.

The chemical entity of the building block comprises at least one reactive group capable of participating in a reaction, which results in a connection between the chemical entity of the building block and another chemical entity or a scaffold associated with the nascent complex. The connection is facilitated by one or more reactive groups of the chemical entity. The number of reactive groups, which appear on the chemical entity, is suitably one to ten. A building block featuring only one reactive group is used i.a. in the end positions of polymers or scaffolds, whereas building blocks having two reactive groups are suitable for the formation of the body part of a polymer or scaffolds capable of being reacted further. One, two or more reactive groups intended for the formation of connections are typically present on scaffolds.

The reactive group of the building block may be capable of forming a direct connection to a reactive group of the nascent complex or the reactive group of the building block may be capable of forming a connection to a reactive group of the nascent complex through a bridging fill-in group. It is to be understood that not all the atoms of a reactive group are necessarily maintained in the connection formed. Rather, the reactive groups are to be regarded as precursors for the structure of the connection.

The subsequent cleavage step to release the chemical entity from the building block can be performed in any appropriate way. In an aspect of the invention the cleavage involves usage of a reagent or an enzyme. The cleavage results in a transfer of the chemical entity to the nascent encoded molecule

or in a transfer of the nascent encoded molecule to the chemical entity of the building block. In some cases it may be advantageous to introduce new chemical groups as a consequence of linker cleavage. The new chemical groups may be used for further reaction in a subsequent cycle, either directly or after having been activated. In other cases it is desirable that no trace of the linker remains after the cleavage.

In another aspect, the connection and the cleavage are conducted as a simultaneous reaction, i.e. either the chemical entity of the building block or the nascent encoded molecule is a leaving group of the reaction. In general, it is preferred to design the system such that the connection and the cleavage occur simultaneously because this will reduce the number of steps and the complexity. The simultaneous connection and cleavage can also be designed such that either no trace of the linker remains or such that a new chemical group for further reaction is introduced, as described above.

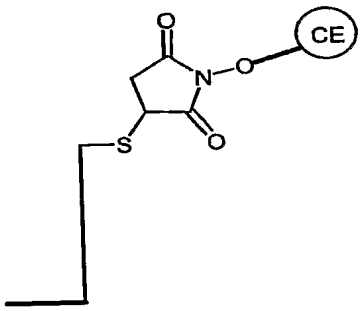
The attachment of the chemical entity to the building block, optionally via a suitable spacer can be at any entity available for attachment, e.g. the chemical entity can be attached to a nucleobase or the backbone. In general, it is preferred to attach the chemical entity at the phosphor of the internucleoside linkage or at the nucleobase. When the nucleobase is used for attachment of the chemical entity, the attachment point is usually at the 7 position of the purines or 7-deaza-purins or at the 5 position of pyrimidines. The nucleotide may be distanced from the reactive group of the chemical entity by a spacer moiety. The spacer may be designed such that the conformational space sampled by the reactive group is optimized for a reaction with the reactive group of the nascent encoded molecule or reactive site.

The anticodon complements the codon of the identifier oligonucleotide sequence and generally comprises the same number of nucleotides as the codon. The anticodon may be adjoined with a fixed sequence, such as a sequence complementing a framing sequence.

Various specific building blocks are envisaged. Building blocks of particular interest are shown below.

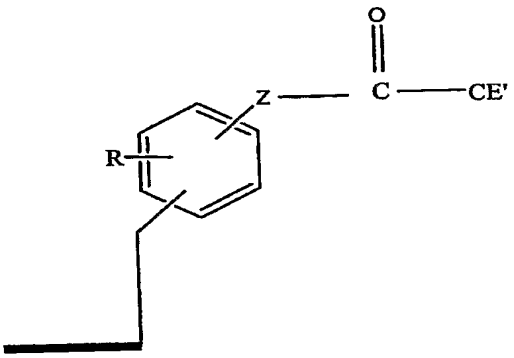
Building blocks transferring a chemical entity to a recipient nucleophilic group

The building block indicated below is capable of transferring a chemical entity (CE) to a recipient nucleophilic group, typically an amine group. The bold lower horizontal line illustrates the building block comprising an anti-codon and the vertical line illustrates a spacer. The 5-membered substituted N-hydroxysuccinimid (NHS) ring serves as an activator, i.e. a labile bond is formed between the oxygen atom connected to the NHS ring and the chemical entity. The labile bond may be cleaved by a nucleophilic group, e.g. positioned on a scaffold



The 5-membered substituted N-hydroxysuccinimid (NHS) ring serves as an activator, i.e. a labile bond is formed between the oxygen atom connected to the NHS ring and the chemical entity. The labile bond may be cleaved by a nucleophilic group, e.g. positioned on a scaffold, to transfer the chemical entity to the scaffold, thus converting the remainder of the fragment into a leaving group of the reaction. When the chemical entity is connected to the activator through a carbonyl group and the recipient group is an amine, the bond formed on the scaffold will an amide bond. The above building block is the subject of WO03078627A2, the content of which is incorporated herein in their entirety by reference.

Another building block, which may form an amide bond, is

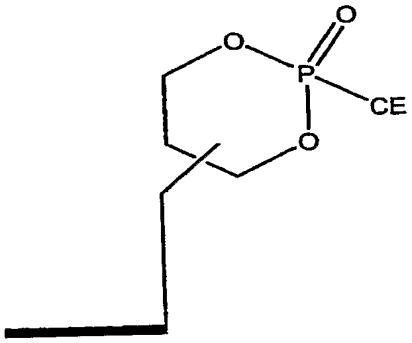


R may be absent or NO_2, CF_3, halogen, preferably Cl, Br, or I, and Z may be S or O. This type of building block is disclosed in WO03078626A2. The content of this patent application is incorporated herein in the entirety by reference.

A nucleophilic group can cleave the linkage between Z and the carbonyl group thereby transferring the chemical entity –(C=O)-CE' to said nucleophilic group.

Building blocks transferring a chemical entity to a recipient reactive group forming a C=C bond

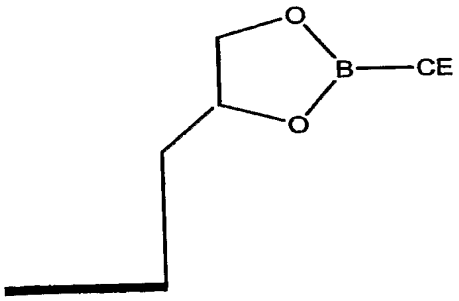
A building block as shown below is able to transfer the chemical entity to a recipient aldehylde group thereby forming a double bond between the carbon of the aldehyde and the chemical entity



The above building block is disclosed in WO03078445A2, the content of which being incorporated herein in the entirety by reference.

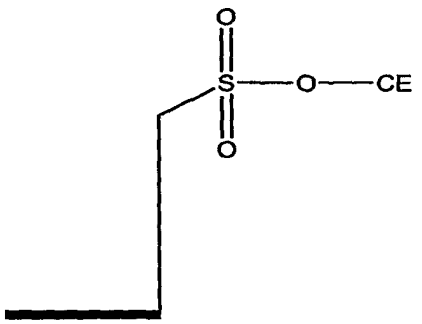
Building blocks transferring a chemical entity to a recipient reactive group forming a C-C bond

The below building block is able to transfer the chemical entity to a recipient group thereby forming a single bond between the receiving moiety, e.g. a scaffold, and the chemical entity.



The above building block is disclosed in WO03078445A2, the content of which being incorporated herein in the entirety by reference.

Another building block capable of transferring a chemical entity to a receiving reactive group forming a single bond is



The receiving group may be a nucleophile, such as a group comprising a hetero atom, thereby forming a single bond between the chemical entity and the hetero atom, or the receiving group may be an electronegative carbon atom, thereby forming a C-C bond between the chemical entity and the scaffold. The above building block is disclosed in WO03078446A2, the content of which is incorporated herein by reference.

The chemical entity attached to any of the above building blocks may be a selected from a large arsenal of chemical structures. Examples of chemical entities are

H or entities selected among the group consisting of a C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-$NR^4{}_2$, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-$NR^4{}_2$, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9.

where R^4 is H or selected independently among the group consisting of C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, heteroaryl, said group being substituted with 0-3 R^9 and

R^5 is selected independently from $-N_3$, -CNO, $-C(NOH)NH_2$, -NHOH, $-NHNHR^6$, $-C(O)R^6$, $-SnR^6{}_3$, $-B(OR^6)_2$, $-P(O)(OR^6)_2$ or the group consisting of C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl said group being substituted with 0-2 R^7,

where R^6 is selected independently from H, C_1-C_6 alkyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-5 halogen atoms selected from -F, -Cl, -Br, and –I; and

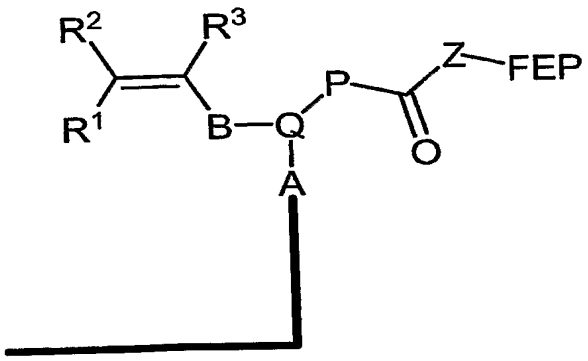
R^7 is independently selected from $-NO_2$, $-COOR^6$, $-COR^6$, -CN, $-OSiR^6_3$, $-OR^6$ and $-NR^6_2$.

R^8 is H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-3 substituents independently selected from –F, -Cl, $-NO_2$, $-R^3$, $-OR^3$, $-SiR^3_3$

R^9 is =O, -F, -Cl, -Br, -I, -CN, $-NO_2$, $-OR^6$, $-NR^6_2$, $-NR^6$-$C(O)R^8$, $-NR^6$-$C(O)OR^8$, $-SR^6$, $-S(O)R^6$, $-S(O)_2R^6$, $-COOR^6$, $-C(O)NR^6_2$ and $-S(O)_2NR^6_2$.

Cross-link cleavage building blocks

It may be advantageous to split the transfer of a chemical entity to a recipient reactive group into two separate steps, namely a cross-linking step and a cleavage step because each step can be optimized. A suitable building block for this two-step process is illustrated below:



Initially, a reactive group appearing on the functional entity precursor (abbreviated FEP) reacts with a recipient reactive group, e.g. a reactive group appearing on a scaffold, thereby forming a cross-link. Subsequently, a cleavage is performed, usually by adding an aqueous oxidising agent such as I_2, Br_2,

Cl_2, H^+, or a Lewis acid. The cleavage results in a transfer of the group HZ-FEP- to the recipient moiety, such as a scaffold.

In the above formula

Z is O, S, NR^4

Q is N, CR^1

P is a valence bond, O, S, NR^4, or a group C_{5-7}arylene, C_{1-6}alkylene, C_{1-6}O-alkylene, C_{1-6}S-alkylene, NR^1-alkylene, C_{1-6}alkylene-O, C_{1-6}alkylene-S option said group being substituted with 0-3 R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-NR^4_2, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-NR^4_2, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9,

B is a group comprising D-E-F, in which

D is a valence bond or a group C_{1-6}alkylene, C_{1-6}alkenylene, C_{1-6}alkynylene, C_{5-7}arylene, or C_{5-7}heteroarylene, said group optionally being substituted with 1 to 4 group R^{11},

E is, when present, a valence bond, O, S, NR^4, or a group C_{1-6}alkylene, C_{1-6}alkenylene, C_{1-6}alkynylene, C_{5-7}arylene, or C_{5-7}heteroarylene, said group optionally being substituted with 1 to 4 group R^{11},

F is, when present, a valence bond, O, S, or NR^4,

A is a spacing group distancing the chemical structure from the complementing element, which may be a nucleic acid,

R^1, R^2, and R^3 are independent of each other selected among the group consisting of H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3 R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-NR^4_2, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-NR^4_2, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9,

FEP is a group selected among the group consisting of H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, and heteroaryl, said group being substituted with 0-3

R^4, 0-3 R^5 and 0-3 R^9 or C_1-C_3 alkylene-NR^4_2, C_1-C_3 alkylene-$NR^4C(O)R^8$, C_1-C_3 alkylene-$NR^4C(O)OR^8$, C_1-C_2 alkylene-O-NR^4_2, C_1-C_2 alkylene-O-$NR^4C(O)R^8$, C_1-C_2 alkylene-O-$NR^4C(O)OR^8$ substituted with 0-3 R^9,

where R^4 is H or selected independently among the group consisting of C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, C_3-C_7 cycloheteroalkyl, aryl, heteroaryl, said group being substituted with 0-3 R^9 and

R^5 is selected independently from -N_3, -CNO, -$C(NOH)NH_2$, -NHOH, -$NHNHR^6$, -$C(O)R^6$, -SnR^6_3, -$B(OR^6)_2$, -$P(O)(OR^6)_2$ or the group consisting of C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_4-C_8 alkadienyl said group being substituted with 0-2 R^7,

where R^6 is selected independently from H, C_1-C_6 alkyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-5 halogen atoms selected from -F, -Cl, -Br, and –I; and R^7 is independently selected from $–NO_2$, -$COOR^6$, -COR^6, -CN, -$OSiR^6_3$, -OR^6 and -NR^6_2.

R^8 is H, C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, C_3-C_7 cycloalkyl, aryl or C_1-C_6 alkylene-aryl substituted with 0-3 substituents independently selected from –F, -Cl, $–NO_2$, -R^3, -OR^3, -SiR^3_3

R^9 is =O, -F, -Cl, -Br, -I, -CN, -NO_2, -OR^6, -NR^6_2, -NR^6-$C(O)R^8$, -NR^6-$C(O)OR^8$, -SR^6, -$S(O)R^6$, -$S(O)_2R^6$, -$COOR^6$, -$C(O)NR^6_2$ and -$S(O)_2NR^6_2$.

In a preferred embodiment Z is O or S, P is a valence bond, Q is CH, B is CH_2, and R^1, R^2, and R^3 is H. The bond between the carbonyl group and Z is cleavable with aqueous I_2.

Contacting between target and library

The contacting step, by which the library of bifunctional molecules is subjected under binding conditions to a target, may be referred to as the enrichment step or the selection step, as appropriate, and includes the screening of the library for display molecules having predetermined desirable characteris-

tics. Predetermined desirable characteristics can include binding to a target, catalytically changing the target, chemically reacting with a target in a manner which alters/modifies the target or the functional activity of the target, and covalently attaching to the target as in a suicide inhibitor.

In theory, display molecules of interest can be selected based on their properties using either physical or physiological procedures. The method preferred according to the present invention is to enrich molecules with respect to binding affinity towards a target of interest. In a certain embodiment, the basic steps involve mixing the library of complexes with the target of interest. The target can be attached to a column matrix or microtitre wells with direct immobilization or by means of antibody binding or other high-affinity interactions. In another embodiment, the target and displayed molecules interact without immobilisation of the target. Displayed molecules that bind to the target will be retained on this surface, while nonbinding displayed molecules in a certain aspect of the invention will be removed during a single or a series of wash steps. The identifier oligonucleotides of complexes bound to the target can then be recovered. It may be considered advantageously to perform a chromatography step after or instead of the washing step, notably in cases where the target is not immobilized. After the recovery of the identifier oligonucleotides they are optionally amplified before the decoding step.

A significant reduction in background binders may be obtained with increased washing volumes, repeating washing steps, higher detergent concentrations and prolonged incubation during washing. Thus, the more volume and number of steps used in the washing procedure together with more stringent conditions the more efficiently the non-binders and background binders will be removed. The right stringency in the washing step can also be used to remove low-affinity specific binders. However, the washing step will also remove wanted binders if too harsh conditions are used.

A blocking step, such as incubation of solid phase with skimmed milk proteins or other inert proteins and/or mild detergent such as Tween-20 and Triton X-100, may also be used to reduce the background. The washing conditions should be as stringent as possible to remove background binding but to retain specific binders that interact with the target. Generally, washing conditions are adjusted to maintain the desired affinity binders, e.g. binders in the micro molar, nanomolar, or picomolar range.

The present invention takes advantages of the fact that the identifier oligonucleotides of low-binding complexes will be in a low concentration compared to the identifier oligonucleotides of complexes binding with high affinity. The generated imbalance can be enhanced in the subsequent formation of a mixture of homo- and hetoro-duplexes and the recovery of homo-duplexes.

The target can be any compound of interest. E.g. the target can be a protein, peptide, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, substrate, metabolite, transition state analogue, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, etc. without limitation. Suitable targets include, but are not limited to, angiotensin converting enzyme, renin, cyclooxygenase, 5-lipoxygenase, IIL- 1 0 converting enzyme, cytokine receptors, PDGF receptor, type II inosine monophosphate dehydrogenase, β-lactamases, integrin, proteases like factor VIIa, kinases like Bcr-Abl/Her, phosphotases like PTP-1B, and fungal cytochrome P-450. Targets can include, but are not limited to, bradykinin, neutrophil elastase, the HIV proteins, including *tat, rev, gag, int,* RT, nucleocapsid etc., VEGF, bFGF, TGFβ, KGF, PDGF, GPCR, thrombin, substance P, IgE, sPLA2, red blood cells, glioblastomas, fibrin clots, PBMCs, hCG, lectins, selectins, cytokines, ICP4, complement proteins, etc.

A target can also be a surface of a non-biological origin, such as a polymer surface or a metal surface. The method of the invention may then be used to

identify suitable coatings for such surfaces.

In a preferred embodiment, the desirable display molecule acts on the target without any interaction between the nucleic acid attached to the desirable encoded molecule and the target. In one embodiment, the bound complex-target aggregate can be partitioned from unbound complexes by a number of methods. The methods include nitrocellulose filter binding, column chromatography, filtration, affinity chromatography, centrifugation, and other well known methods. A preferred method is size-exclusion chromatography.

Briefly, the library of complexes is subjected to the target, which may include contact between the library and a column onto which the target is immobilised. Identifier oligonucleotides associated with undesirable display molecules, i.e. display molecules not bound to the target under the stringency conditions used, will pass through the column. Additional undesirable display molecules (e.g. display molecules which cross-react with other targets) may be removed by counter-selection methods. Desirable complexes are bound to the column. The target may be immobilized in a number of ways. In one embodiment, the target is immobilized through a cleavable physical link, such as one more chemical bonds.

The complex may be provided with a cleavable linker at a position between the display molecule and the identifier oligonucleotide. When the target is immobilized, the cleavable linker of the complex is preferable orthogonal to the cleavable linker that attached the target to the solid support. The cleavable linker may be cleaved to separate the identifier oligonucleotides of complexes having affinity towards the targets. Just to mention a single type of orthogonal cleavable linkages, one could attached to target to the solid support through a linkage that can be cleaved by a chemical agent, and the linker separating the display molecule and the identifier oligonucleotide may be selected as a photocleavable linkage. More specifically, the former linkage

may be a disulphide bond that can be cleaved by a suitable reducing agent like DTT (dithiothreitol) and the latter linkage may be an *o*-nitrophenyl group.

There are other partitioning and screening processes which are compatible with this invention that are known to one of ordinary skill in the art. Such known process may be used in combination with the present inventive method. In one embodiment, the complex-target aggregate can be fractionated by common methods and then each fraction is assayed for activity. The fractionization methods can include size, pH, hydrophobicity, etc.

Inherent in the present method is the selection of encoded molecules on the basis of a desired function; this can be extended to the selection of molecules with a desired function and specificity. Specificity can be required during the selection process by first extracting complexes which are capable of interacting with a non-desired "target" (negative selection, or counter-selection), followed by positive selection with the desired target. As an example, inhibitors of fungal cytochrome P-450 are known to cross-react to some extent with mammalian cytochrome P-450 (resulting in serious side effects). Highly specific inhibitors of the fungal cytochrome could be selected from a library by first removing those complexes capable of interacting with the mammalian cytochrome, followed by retention of the remaining products which are capable of interacting with the fungal cytochrome.

In a certain embodiment, a binding platform may be constructed that can be used for almost any target. The binding platform should preferably be small enough to only allow association of a few or a single target molecule. This to ensure a solution based selection procedure with adjustable target concentration. The binding platform is primarily composed of two components; a small surface allowing association of the target molecule, and an association area/site for the target oligonucleotide. This binding platform may be designed to mediate the association of the target and target oligonucleotide to allow proximity selection in solution.

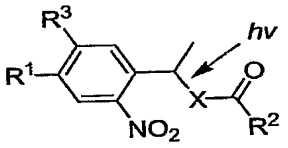
Cleavable linkers

A cleavable linker may be positioned between the target and a solid support, between the display molecule and the identifier oligonucleotide, or any other position that can provide for a separation of the identifier oligonucleotides of successful complexes from non-specific binding complexes. The cleavable linker may be selectively cleavable, i.e. conditions may selected that only cleave that particular linker.

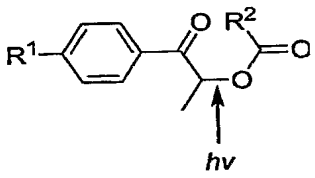
The cleavable linkers may be selected from a large plethora of chemical structures. Examples of linkers include, but are not limited to, linkers having an enzymatic cleavage site, linkers comprising a chemical degradable component, and linkers cleavable by electromagnetic radiation, such as light.

Examples of linkers cleavable by electromagnetic radiation (light)

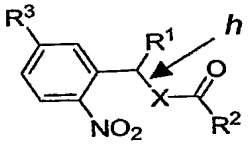
o-nitrobenzyl



p-alkoxy

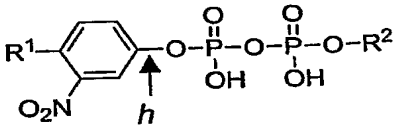


o-nitrobenzyl in exo position



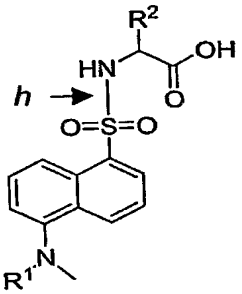
For more details see Holmes CP. J. Org. Chem. **1997,** *62*, 2370-2380

3-nitrophenyloxy



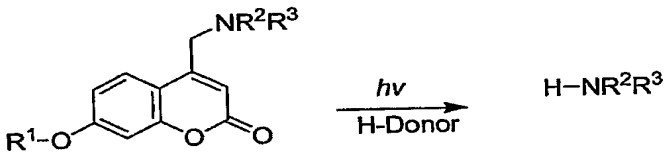
For more details see Rajasekharan Pillai, V. N. Synthesis. **1980**, 1-26

Dansyl derivatives:



For more details see Rajasekharan Pillai, V. N. Synthesis. **1980**, 1-26

Coumarin derivatives



For more details see R. O. Schoenleber, B. Giese. Synlett **2003**, 501-504

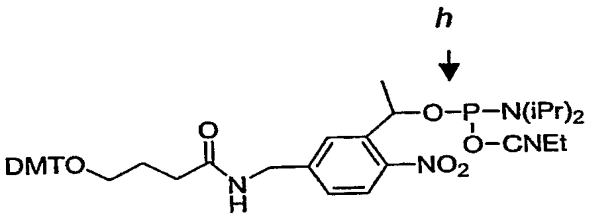
R^1 and R^2 can be either of the potential drug candidate and the identifier oligonucleotide, respectively. Alternatively, R^1 and R^2 can be either of the target or a solid support, respectively.

R^3 = H or OCH_3

If X is O then the product will be a carboxylic acid

If X is NH the product will be a carboxamide

One specific example is the PC Spacer Phosphoramidite (Glen research catalog # 10-4913-90) which can be introduced in an oligonucleotide during synthesis and cleaved by subjecting the sample in water to UV light (~ 300-350 nm) for 30 seconds to 1 minute.

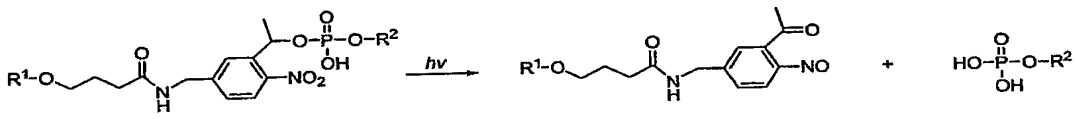


DMT = 4,4'-Dimethoxytrityl

iPr = Isopropyl

CNEt = Cyanoethyl

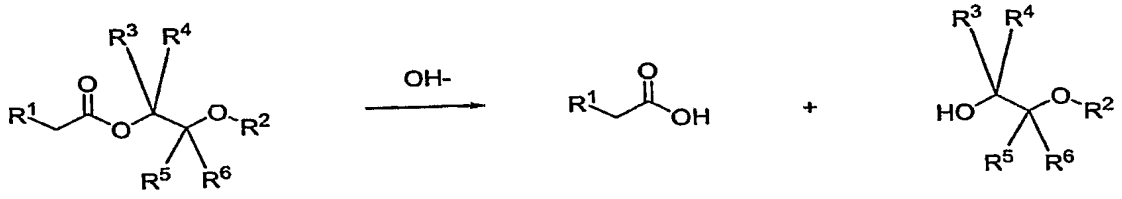
The above PC spacer phosphoamidite is suitable incorporated in a library of complexes at a position between the indentifier and the potential drug candidate. The spacer may be cleaved according to the following reaction.



R^1 and R^2 can be either of the encoded molecule and the identifying molecule, respectively. In a preferred aspect R^2 is an oligonucleotide identifier and the R^1 is the potential drug candidate. When the linker is cleaved a phosphate group is generated allowing for further biological reactions. As an example, the phosphate group may be positioned in the 5´end of an oligonucleotide allowing for an enzymatic ligation process to take place.

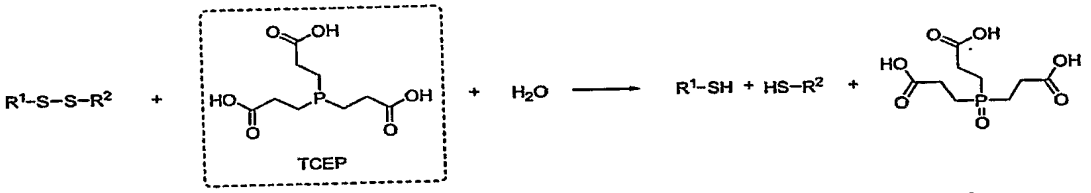
Examples of linkers cleavable by chemical agents:

Ester linkers can be cleaved by nucleophilic attack using e.g. hydroxide ions. In practice this can be accomplished by subjecting the target-ligand complex to a base for a short period.



R^1 and R^2 can be the either of be the potential drug candidate or the identifier oligonucleotide, respectively. R^{4-6} can be any of the following: H, CN, F, NO_2, SO_2NR_2.

Disulfide linkers can efficiently be cleaved / reduced by Tris (2-carboxyethyl) phosphine (TCEP). TCEP selectively and completely reduces even the most stable water-soluble alkyl disulfides over a wide pH range. These reductions frequently required less than 5 minutes at room temperature. TCEP is a non-volatile and odorless reductant and unlike most other reducing agents, it is resistant to air oxidation. Trialkylphosphines such as TCEP are stable in aqueous solution, selectively reduce disulfide bonds, and are essentially unreactive toward other functional groups commonly found in proteins.



More details on the reduction of disulfide bonds can be found in Kirley, T.L.(1989), Reduction and fluorescent labeling of cyst(e)ine-containing proteins for subsequent structural analysis, *Anal. Biochem.* **180**, 231 and Levison, M.E., *et al.* (1969), Reduction of biological substances by water-soluble phosphines: Gamma-globulin. *Experentia* **25**, 126-127.

Linkers cleavable by enzymes

The linker connecting the potential drug candidate with the identifier oligonucleotide or the solid support and the target can include a peptide region that allows a specific cleavage using a protease. This is a well-known strategy in molecular biology. Site-specific proteases and their cognate target amino acid sequences are often used to remove the fusion protein tags that facilitate enhanced expression, solubility, secretion or purification of the fusion protein.

Various proteases can be used to accomplish a specific cleavage. The specificity is especially important when the cleavage site is presented together with other sequences such as for example the fusion proteins. Various conditions have been optimized in order to enhance the cleavage efficiency and control the specificity. These conditions are available and know in the art.

Enterokinase is one example of an enzyme (serine protease) that cut a specific amino acid sequence. Enterokinase recognition site is Asp-Asp-Asp-Asp-Lys (DDDDK), and it cleaves C-terminally of Lys. Purified recombinant Enterokinase is commercially available and is highly active over wide ranges in pH (pH 4.5-9.5) and temperature (4-45°C).

The nuclear inclusion protease from tobacco etch virus (TEV) is another commercially available and well-characterized proteases that can be used to cut at a specific amino acid sequence. TEV protease cleaves the sequence Glu-Asn-Leu-Tyr-Phe-Gln-Gly/Ser (ENLYFQG/S) between Gln-Gly or Gln-Ser with high specificity.

Another well-known protease is thrombin that specifically cleaves the sequence Leu-Val-Pro-Arg-Gly-Ser (LVPAGS) between Arg-Gly. Thrombin has also been used for cleavage of recombinant fusion proteins. Other sequences can also be used for thrombin cleavage; these sequences are more or less specific and more or less efficiently cleaved by thrombin. Thrombin is

a highly active protease and various reaction conditions are known to the public.

Activated coagulation factor FX (FXa) is also known to be a specific and useful protease. This enzyme cleaves C-terminal of Arg at the sequence Ile-Glu-Gly-Arg (IEGR). FXa is frequently used to cut between fusion proteins when producing proteins with recombinant technology. Other recognition sequences can also be used for FXa.

Other types of proteolytic enzymes can also be used that recognize specific amino acid sequences. In addition, proteolytic enzymes that cleave amino acid sequences in an un-specific manner can also be used if only the linker contains an amino acid sequence in the complex molecule.

Other type of molecules such as ribozymes, catalytically active antibodies, or lipases can also be used. The only prerequisite is that the catalytically active molecule can cleave the specific structure used as the linker, or as a part of the linker, that connects the encoding region and the displayed molecule or, in the alternative the solid support and the target.

A variety of endonucleases are available that recognize and cleave a double stranded nucleic acid having a specific sequence of nucleotides. The endonuclease Eco RI is an example of a nuclease that efficiently cuts a nucleotide sequence linker comprising the sequence GAATTC also when this sequence is close to the nucleotide sequence length. Purified recombinant Eco RI is commercially available and is highly active in a range of buffer conditions. As an example the Eco RI is working in in various protocols as indicted below (NEBuffer is available from New England Biolabs):
NEBuffer 1 : [10 mM Bis Tris Propane-HCl, 10 mM MgCl2, 1 mM dithiothreitol (pH 7.0 at 25°C)],
NEBuffer 2 : [50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM dithiothreitol (pH 7.9 at 25°C)],

NEBuffer 3 : [100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM dithiothreitol (pH 7.9 at 25°C)],
NEBuffer 4 : [50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9 at 25°C)].
Extension buffer : mM KCl, 20 mM Tris-HCl(Ph 8.8 at 25o C), 10 mM (NH4)2 SO4 , 2 mM MgSO 4 and 0.1% Triton X-100, and 200 µM dNTPs.

Formation of homo-duplexes

After the identifier oligonucleotides of complexes comprising display molecules interacting in a certain desired fashion with a target are partitioned, a nucleic acid amplification is usually conducted. The nucleic acid amplification method may be PCR or a method aquivalent thereto. The amplification is preferably conducted such that the relative destribution of the individual identifier oligonucleotides are retained. According to a certain embodiment, identical PCR priming sites to obtain a proportional amplification of the identifier oligonucleotides surround the coding section.

After the formation of duplexes, a denaturing step usually follows. A denaturing can be obtained by a variety of methods, including increased temperature, high salt concentration, presence of organic solvents, certain duplex disrupting chemicals, etc. Generally it is preferred to use an elevated temperature. When the temperature is increased above the melting temperature of the duplex the single stranded oligonucleotides are formed. At the elevated temperature, single stranded oligonucleotides complementing the partitioned identifier oligonucleotides optionally may be added.

Homo-duplexes are formed by subjecting the single stranded mixture to hybridisation conditions. When the denaturing is obtained by elevating the temperature, the hybridisation conditions may appropriately be obtained by decreasing the temperature below the melting temperature of the homo-duplex. The temperature decrease rate may be adjusted according to the specific conditions used to obtain the optimal condition for the formation of the homo-

duplexes. If a high temperature decrease rate is selected a lower tendency of homo-duplex formation is obtained. Conversely, a low temperature decrease rate implies a less tendency of hetero-duplex formation. Depending on the renaturing conditions used, predominately homo-duplexes or a mixture of homo- and hetero-duplexes are formed. When the denaturing is obtained using hybridisation modifying agents, like salt, solvents etc, hybridisation conditions may be obtained by desalination in case of salt and evaporation in case of solvents.

As mentioned above, the mixture of denatured single stranded oligonucleotides may be added complementing oligonucleotides to obtain certain desired effects. In an aspect of the invention random oligonucleotides are added to the single stranded oligonucleotides to lower the general tendency of duplex formation. A less tendency may be desirable because the selectivity for the best binding display molecule increases. In another aspect of the invention, certain specific complementing oligonucleotides are added to increase the probability of duplex formation for certain kind of identifier oligonucleotides. The added oligonucleotides may be immobilized or capable of being immobilized on a solid support. Alternatively, the addition of certain complementing oligonucleotides may compensate for an initial library not having an even distribution of the concentration of individual members.

In certain applications of the invention it is preferred to use amplification of only one strand, thereby to produce single stranded identifier oligonucleotides. The single stranded identifier oligonucleotides may then be mixed with a mixture of oligonucleotides complementing the original oligonucleotides of the library. The more frequent abundant single stranded identifier oligonucleotides will be more inclined to form a higher portion of homo-duplex compared to the less frequently occurring, which will tend to be in single stranded form or in hetero-duplex form. It may be desired to spike the partitioned fraction of single stranded identifier oligonucleotides with a higher concentration of certain complementing oligonucleotides in order to bias the formation of

homo- and hetero-duplexes. Alternatively, certain complementing oligonucleotides may be added in a lower amount. As an example, the identifier oligonucleotide coding for a known ligand may be extinguished because it is desired to find unknown ligands, by avoiding the addition of an oligonucleotide complementing said identifier oligonucleotide.

In some aspects of the invention, a library of different display molecules, each being associated with an identifying oligonucleotide, is divided into two or more portions. A first portion may be contacted with a target and the identifier oligonucleotides of successful display molecules harvested, while a second portion may be contacted with a blank vessel or a second target and the identifier oligonucleotides of successful display molecules of the second portion collected. Prior to the step of denaturing and renaturing, the identifier oligonucleotides from the two portions are mixed. The advantages of screening two or more portions of a library individually include changing the profile of the background, obtaining subtype selectivity of the display molecule etc. It is also possible to combine two or more libraries before the contacting with the target to obtain an altered profile of binding display molecules.

Recovery of homo-duplexes

The recovery of homo-duplex is typically obtained by removing the hetero-duplexes and the single stranded oligonucleotides from the renaturate mixture. In another aspect of the invention, the homo-duplexes are extracted form the mixture. When the hetero-duplexes are removed from the reaction mixture, any convenient method can be used, such as a mis-match binding, enzymatic or chemical mis-match cleavage, or physical method.

Mis-match binding

In a certain aspect of the invention, hetero-duplexes are removed by binding to prokaryotic or eukaryotic mis-match binding proteins. An example is MutS, a mismatch binding protein isolated from E. coli, which recognises regions of double-stranded DNA containing a mismatched base pair (Wagner el al.,

1995, Nucleic Acids Research, 22, 1541-1547) as well as 1 to 4 base pair insertion-deletion loops. MutS is allowed to bind to the hetero-duplexes and bound hetero-duplex/MutS complexes are removed from the reaction mixture using, for example, powdered nitrocellulose. A convenient alternative is to use MutS conjugated to magnetic beads, allowing bound heteroduplexes to be removed from the reaction mixture with a magnet. MutS may also be conjugated to biotin and the bound hetero-duplexes removed from the mixture using streptavidin-coated beads.

MutY has considerable potential for mismatch detection, as its *in vivo* function is to repair mismatched G:A base pairing by cleavage of the adenine-containing strand. Similar proteins thought to be involved in G:T and G:U mismatch repair have also been described. Hsu (Carcinogenesis 1994;15:1657-62) described the use of *E. coli* MutY protein for the detection of mismatched G:A in p53. As low as 1-2% mutant DNA in a sample of mutant and wild-type DNA could be detected using a synthetic DNA oligonucleotide to create G/A mis-pairing.

Enzymatic cleavage

Chemical and enzymatical cleavage methods used for degradation of hetero-duplexes must differentially cleave these sequences and retain the homo-duplexes. Any sequence difference will result in the formation of a mispairing, causing localized distortion of the double helix. Cleavage techniques exploit this structural change by selectively degrading or modifying DNA at the site of the mismatch. Ideally, little or no cleavage would be seen in a perfectly matched DNA fragment, and all distortions of the helix generated by base mismatches would result in cleavage. In practice neither criteria a fully met, and the utility of a technique becomes a trade-off between ease of use, sensitivity and specificity.

In certain aspect of the invention mammalian or bacterial endonucleases are used to recognise and cleave the hetero-duplexes at mismatched nucleo-

bases (see U.S. Pat. No. 5,824,471). Examples of preferred enzymes include bacteriophage resolvases such as T4 endonuclease VII or T7 endonuclease I. In a preferred aspect of the invention, thermostable cleavage enzymes would be used in order to avoid the necessity of adding fresh enzyme during each round of heteroduplex formation and removal.

An enzyme called "Cleavase" from Third Wave Technologies relies on the endonucleolytic cleavage of stem-loop structures. The precise nature of the thermostable enzyme "Cleavase" has not been published. Since the stem-loop profile is dependent upon the primary sequence of the DNA, sequence changes in some cases result in a change in the cleavage profile. This method is unique in that unlike all other enzymatic techniques, it does not require the formation of a mismatch heteroduplex to generate a site for enzymatic cleavage.

Ribonuclease A cleavage was originally described by Myers *et al* (Science 1985; 230:1242-6) using DNA:RNA hybrids. Sensitivity was reported to be around 60% per strand cleaved. Grange *et al* (Nucleic Acids Research 1990; 18:4227-36) described improved sensitivity by screening both strands of RNA. A Non-Isotopic RNase Cleavage Assay (NIRCA) has also been described. This assay (commercially available from Ambion) utilized PCR primers with phage RNA polymerase promoters so that large quantities of RNA were produced. Cleaved products could be detected on agarose gels and fragments of up to 1 kilobase were analyzed. Additional RNase enzymes such as RNase 1 and RNase T1 increase the sensitivity of the assay. The commercial kit includes a helix modifying reagent that makes the mismatches more sensitive to cleavage.

T4 endonuclease VII (T4E7) and T7E1 are small proteins from bacteriophages that bind as homodimers and cleave aberrant DNA structures including Holliday Junctions (and are hence sometimes called "resolvases") though it is far from clear that they perform such a role *in vivo.* Mashal *et al* (Nature

Genetics 1995; 9:177-83) observed that they preferentially cleaved mismatched hetero-duplexes, leading to the possibility of an enzymatic equivalent to the chemical cleavage of mismatch. DNA requires no special preparation after amplification like GC clamping or including primers with 'phage promoters. Background peaks which are seen are highly reproducible and may therefore amenable to background subtraction algorithms like those applied to DNA sequencing traces. A commercially available T4E7-based mutation detection kit is available from Amersham-Pharmacia.

A plant endonuclease (CEL I) with similar activity has also been described. CEL I is one of series of plant endonucleases with similar activity to nuclease S1 but at neutral pH instead of pH 4 or 5. Like T4E7, the cleavage efficiency varies according to the mismatch examined and background cleavage is dependent on the template being examined.

Uracil glycosylase and photo-activated guanine modification reagent have been used to develop a cleavage method that essentially produces T or G sequencing tracks. DNA synthesis by PCR requires the incorporation of a proportion of uracil bases in place of thymines. These can be removed by uracil glycosylase and the abasic site then cleaved by heat or enzymatic treatment.

The present invention also includes any combination of enzymes.

Chemical method

Chemical cleavage of mismatches was developed as a modification of the Maxam-Gilbert DNA sequencing method by Cotton *et al* (Proc Natl Acad Sci (US) 1988;85: 4397-401). Mismatched thymines are susceptible to modification by osmium tetroxide (or potassium permanganate and tetraethyl ammonium acetate) and mismatched cytosines can be modified by hydroxylamine. The modified bases are then cleaved by hot piperidine treatment.

A chemical method can also be used for preventing a PCR amplification to be performed. The chemical methods includes DMS-modification, ketoxal-modification, DEPT-modification etc. and take advantage of the fact that a single stranded oligonucleotide is more volnable to chemical reaction than the corresponding oligonucleotide in double stranded form. The chemical methods usually result in a modification of a nucleobase so that a polymerase cannot recognise the oligonucleotide as a substrate and therefore cannot perform a PCR amplification. When a PCR amplification is performed prior to step d only identifier oligonucleotides of homo-duplexes is amplified while single stranded and hetero-duplexes will be repressed.

Physical method

The physical method of recovering homo-duplex molecules involves physical separation, such as achieved by chromatography or electrophoresis. Suitable chromatography methods include column chromatography, affinity chromatography, size-exclusion chromatography and gel chromatography. Suitable gels for gel chromatography include non-denaturing gels, such as agarose or polyacrylamide gels. The chromatography can be performed at elevated temperatures, e.g. above ambient temperatures, to favour the formation of homo-duplexes. In a certain aspect of the invention, the temperature is selected between the average melting temperature of the homo-duplexes and the melting temperature of the hetero-duplexes having the least amount of mis-matches. Generally, a temperature for performing gel chromatography is selected in the range of 45 to 80 degrees Celsius.

Suitable physical methods notably include denaturing high performance liquid chromatography (DHPLC) and chemical or temperature denaturing electrophoresis. Denaturing HPLC is a chromatographic technique capable of separating homo-duplex DNA molecules from a mixture of hetero-duplex and single stranded oligonucleotides. The mixture is applied to a stationary reverse-phase support and the homo- and hetero-duplex molecules are eluted (under thermal or chemical conditions capable of at least partially denaturing hetero-

duplexes) with a mobile phase containing an ion-pairing reagent (e.g. triethylammonium acetate; TEAA) and an organic solvent (e.g. acetonitrile; AcN). DHPLC can also allow the direct quantisation of relative homo-duplex and hetero-duplex concentrations by the detection of ultraviolet absorbance or fluorescent emission of/from the separated species. The area under the absorbance/emission peak is proportional to the amount of product, which therefore allows quantitative assessment of the relative proportions. DHPLC is described in Liu W et al. (Nucleic Acids Research. 26:1396-1400, 1998 and O'Donovan MC et al. Genomics. 52:4449, 1998).

A preferred method for use in the instant invention to separate hetero-duplex and homo-duplex molecules is as described in U.S. Pat. No. 5,795,976, which is incorporated herein by reference.

Determining the identifier oligonucleotide sequence

The nucleotide sequence of the identifier sequence is determined to identify the identity of the binding display molecule(s). In a certain embodiment of the invention, chemical entities that participated in the formation of the display molecules that binds to the target are identified. The synthesis method of the display molecule may be established if information on the chemical entities as well as the point in time they have been incorporated in the display molecule can be deduced from the identifier oligonucleotide. It may be sufficient to obtain information on the chemical structure of the various chemical entities that have participated in the formation of the display molecule to deduce the full molecule due to structural constraints during the formation. As an example, the use of different kinds of attachment chemistries may ensure that a chemical entity can only be reacted at a single position on a scaffold. Another kind of chemical constrains may be present due to steric hindrance on the scaffold molecule or the chemical entity to be transferred. In general however, it is preferred that information can be inferred from the identifier oligonucleotide sequence that enable the identification of each of the chemical entities that have participated in the formation of the encoded molecule along

with the point in time in the synthesis history the chemical entities have been incorporated in the (nascent) display molecule.

Although conventional DNA sequencing methods are readily available and useful for this determination, the amount and quality of isolated bifunctional molecules may require additional manipulations prior to a sequencing reaction.

Where the amount is low, it is preferred to increase the amount of the oligonucleotide sequences by polymerase chain reaction (PCR) using PCR primers directed to primer binding sites present in the identifier oligonucleotide sequence.

In one embodiment, the different identifier oligonucleotide sequences are cloned into separate sequencing vectors prior to determining their sequence by DNA sequencing methods. This is typically accomplished by amplifying the different identifier oligonucleotide sequences by PCR and then using a unique restriction endonuclease sites on the amplified product to directionally clone the amplified fragments into sequencing vectors. The cloning and sequencing of the amplified fragments then is a routine procedure that can be carried out by any of a number of molecular biological methods known in the art.

Alternatively, the bifunctional complex or the PCR amplified identifier oligonucleotide sequence can be analysed in a microarray. The array may be designed to analyse the presence of a single codon or multiple codons in a identifier oligonucleotide sequence.

Another approach, the identifier oligonucleotide product is analysed by QPCR. Preferably, the QPCR affords information as to the chemical moieties that has participated in the formation of the display molecules. The QPCR approach also allows a direct investigation of the enrichment factor if two

samples are analysed in parallel, said samples being collected before and after the use of the present method. Various conditions can be investigated to obtain the most optimal selection procedure before the sequences are analysed to identify the precise structures of the binding molecules.

Sequencing can also be performed using pyrosequencing chemistry. A still further method for decoding the identifier oligonucleotides comprises high throughput sequencing, using single molecule approach.

According to the invention, the homo-duplexes are used to deduce the identity of the display molecule(s) interacting with a target. The homoduplexes may be used directly from the recovery step or modified by any biotechnological technique prior to decoding. Notably, the modification can include total or partial amplification of the homo-duplexes to produce a double stranded or single stranded product. Also the modification may include fragmentation, e.g. digestion by a restriction enzyme or another nucleic acid active enzyme. In a certain embodiment, a restriction site is positioned between codons to allow for a separation of codons of the identifier oligonucleotides. The fragmentation may facilitate the subsequent decoding, as small nucleic acids usually are easier to decode.

The recovered duplexes of step e may one or more times be recycled to step d. The recycling may reduce the diversity at the nucleic acid level and at the same time increase the probability that an identifier oligonucleotide from a display molecule interacting with a target is identified when sequencing a limited number of homo-duplexes. Usually, when two or more sequences of a single identifier are detected during the sequencing, this is an indication that a display molecule performing an interaction with the target is identified. The repetition of the hetero- and homo-duplexes formation and recovery of the homo-duplexes may be conducted a suitable number of times until two or more identifier oligonucleotides in a sequencing step of a limited number of homo-duplexes is revealed.

In a certain embodiment of the invention, therefore, a decoding of the homo-duplex is effected prior to a consecutive repetition. The information of the decoding step may be used to modify the composition of the identifier oligonucleotides and strands complementary thereto. Notably, the composition can be modified by removing certain identifier oligonucleotides from the pool. As an example, identifiers of known display molecules interacting with a target can be removed to reveal other display molecules in the library having an ability to perform the same interaction albeit, to a lesser extend.

Various methods for excluding certain identifier oligonucleotides are available, including removal by an immobilized probe, digestion with a sequence specific nuclease etc. An appealing method includes that the partitioned fraction of identifier oligonucleotides is split in two portions. A first portion is then treated as described above to recover homo-duplexes. The recovered homo-duplexes are used to treat the second portion. In a certain aspect of the invention it is preferred to amplify the recovered homo-duplexes using primers conjugated to biotin. When the amplification product containing biotin is mixed with the second portion of the partitioned identifiers under denaturing conditions and subsequently subject to at least partly renaturing conditions, the strands with attached biotin may anneal with a complement in the mixture. A subsequent treatment with streptavidin or avidin allows the biotin labelled duplexes selectively to be removed from the mixture.

BREIF DESRIPTION OF THE DESCRIPTION

Fig. 1 discloses a schematic representation of a selection process.

Fig. 2 depicts a homo- and a hetero-duplex.

Fig. 3 shows a library treated in accordance with the method of the invention.

Fig. 4 discloses the overall principle of mis-match selection.

DETAILED DISCLOSURE OF THE FIGURES

Figure 1 discloses the common selection process in schematic form. In diagram A an ideal library is shown that comprises a variety of different members of a library (Diversity) in the same concentration, i.e. with the same number of each bifunctional complex in the mixture.

The library represented in diagram A is subjected to a selection process. Generally, the selection process implies that the library is contacted with a target to allow for an interaction, usually a binding interaction, to take place. Ideally, the non-binding members of the library are discharged and the bifunctional complexes able to perform an interaction with the target are selected. However, in reality a certain amount of non-binding complexes are eluted together with the binding complexes. The non-binding complexes are referred to a background in diagram B. The background usually increases with the diversity, i.e. a large library generates a higher background relative to small library.

To increase the probability of finding a ligand, it is generally desired to apply a library as large as possible. The high background however generates a high level of noise so that a detection of the hits is difficult or even impossible. The present invention suggests a method to reduce the background so as to be able to identify the hit. In diagram C the background is broken down to individual molecules to illustrate that the amount of the binding ligand is higher than each of the molecules in the selected library. The imbalance formed due to the selection process between the hit and the remainder of the library members is then used in the subsequent steps of the present method.

Fig. 2 schematically discloses a homo-duplex and a hetero-duplex. A homo-duplex is an identifier oligonucleotide hybridised to a fully complementing oligonucleotide. A hetero-duplex is an identifier oligonucleotide hybridised to an oligonucleotide showing one, two, or more mis-matching nucleotides. A mis-matching nucleotide is a nucleotide not paired in accordance with the

Watson-Crick base-pairing rules, in which A pairs with T (or U) and C pairs with G. In Fig. 2, the mis-matching nucleotide(s) are illustrated by a filled circle.

The homo- and hetero-duplexes may be formed by denaturing a PCR product of the result of the selection shown on Fig. 1 and subsequently allow the mixture to hybridise again. The denaturing is usually performed by heating to a temperature above the melting point of the PCR product. The hybridisation conditions are usually obtained by lowering the temperature well below the melting point of the homo-duplexes.

Fig. 3 illustrates various steps of the present invention. In a first step the library is subjected to a selection process as described in Fig. 1, in which the straight line depicts the identifier oligonucleotide from the complex of the binding display molecule. The output of the selection process is in a subsequent step subjected to a melting and reannealing step. Due to the excess of identifier oligonucleotides from the binding display molecule, the mixture of homo- and hetero-duplexes comprises a relatively higher content of homo-duplexes from the identifiers of the binding display molecules than homo-duplexes from other sources. In the final step shown on Fig. 3, the homo-duplexes are separated from the mixture, e.g. by cleavage of hetero-duplexes with an enzyme. The result is an enrichment of identifier oligonucleotides from binding display molecules.

Fig. 4 discloses a diagram of the overall principle of mis-match selection. Initially, a library of bifunctional molecules is subjected to a selection process. According to the broadest scope of the invention, any type of bifunctional complex library can be used, including phage display, ribosome display, and small molecule display. Following the selection, the identifier oligonucleotide is amplified using PCR or a similar method in order to generate more copies of the individual identifier oligonucleotides. Preferably, the amplification retains the proportion between the individual identifier oligonucleotides.

A number of times the following cycle can be repeated: The PCR amplicons are heated to a temperature above the melting point of the homo-duplexes and subsequent cooled to form a mixture of hetero-duplexes and homo-duplexes. The homo-duplexes are recovered from the mixture by i) binding the mis-matched duplexes to a protein, such as MutS, ii) cleaving the hetero-duplex using an appropriate enzyme, such as Cel I, or iii) physical means, such as DHPLC. If the result of the mis-match selection still comprises too much noise, i.e. it is not possible when sequencing a small amount of sequences to deduce one or more sequences, which occurs more frequently than others, the cycle may be repeated, starting with a PCR amplification of the output of the mis-match selection.

The final step of the method includes an analysis of the output. A variety of techniques are available to the skilled person in the art, including sequencing using capillary electrophoresis, bead array, high-density microarray etc. If the mis-match selection has been successful, an analysis of a relatively few oligonucleotides will reveal which display molecules of the library that have the highest binding affinity, because the identifier oligonucleotides of complexes having display molecules with high binding affinity occurs more frequent.

EXAMPLES

EXAMPLE 1. Statistical calculations of mismatch selection (MISE) treatment in a library after initial selection.

Theoretical procedure:

This example describes the statistical calculation of MISE treatment simulating various libraries with certain size and diversity and different enrichment factors in the initial selection process. Below is definition of the parameters used in the calculations.

Steps (x):

1 : initial library

2 : library after selection

3 : library after first MISE round

4 : library after second MISE round

5 : library after third MISE round

6 : library after fourth MISE round

N(t,x) : Total number of molecules in the library in step x

N(p,x) : Total number of molecules in pool p in step x

A(t,x) : Total number of molecules formed in PCR amplification in step x

D(t,x) : Diversity (number of different molecules in library) in step x

D(p,x) : Diversity of pool p (number of different molecules in library) in step x

R(p) : Relative enrichment factor of pool p

SO(x) : Fraction of homoduplexes surviving MISE treatment

SE(x) : Fraction of heteroduplexes surviving MISE treatment

STEP 1: Selection.

In this step the initial library is contacted with target. Unbound templates are washed off and bound templates are recovered. The templates will be recovered according to the binding efficiency of their displayed ligand to the target.

$$N(p,1) = N(p,s) * R(p,s)$$

STEP 2: Calculation of diversity after selection.

The selection step can reduce diversity of the library if for some of the background binders less than 1 molecule survives selection.

If N(p,1) is less than D(p,s) then N(p,1) is set equal to D(p,s) [loss of diversity]

STEP 3: PCR Amplification of selection output.

By addition of a known amount of primers in the PCR amplification, the total amount of molecules in the library after amplification can be normalized to a specific amount [A(t,1)]

After amplification $N(t,1)_{[post\text{-}amplification]}$ equals A(t,1)

And $N(p,1)_{[post\text{-}amplification]}$ equals $N(p,1)_{[pre\text{-}amplification]} * A(t,1) / N(t,1)_{[pre\text{-}amplification]}$

STEP 4: Homo- and hetero-duplex separation.

The resulting duplexes are denatured by heating and the individual strands are reannealed in a random fashion. Thus the re-formation of duplexes takes place stochastically.

The frequency of a species in the total library is calculated by

F(p,1) = N(p,1) / N(t,1)

Since all species in a pool behave in the same fashion in this simulation, the frequency is equal for all species in a pool.

The number of homoduplexes formed in pool [p] is

O(p,1) = [N(t,1) * (F(p,1))^2 * D(p,1)] / 2

The number of heteroduplexes involving strands from pool [p] is

E(p,1) = 2 * F(p,1) * (1-F(p,1)) * D(p,1) * N(t,1)

STEP 5: Homo- and hetero-duplex separation.

All duplexes (homo- and heteroduplexes) are reacted with a mismatch-specific enzyme that specifically degrades heteroduplexes. It is expected that a small fraction of homoduplexes is also degraded by the mismatch-specific enzyme.

The number of surviving homoduplexes in pool p is

O(p,1) = SO (1)

If O(p,1) is then less than zero O(p,1) is set to zero [less than one molecule exists]

The number of surviving heteroduplexes in pool p is

E(p,1) = SE (1)

If O(p,1) is then less than zero O(p,1) is set to zero

STEP 6: Amplification

The total library is normalized to N(t,2) corresponding to a PCR step.

Fraction in pool p of number of total molecules:

Calculated example A

A model library composed of 5 pools (b-f) that contain species [pool b contains 16 species, pool c contains 8 species etc.] that bind target specifically and can be enriched from 0,1 fold (pool f) to 6,25e-3 fold (pool b). Pool a contains 1e8 species (minus the sum of the other pools) that bind unspecifically and therefore are depleted relatively from the library during selection: R(a) = 1e-7. In this case the selection step reduces the number of molecules in pool a from approximately 1e11 to 1e11*R(a) = 1e4 and the number of molecules in pool f from 1000 to 100.

N(t,x) = 1e11 : Total number of molecules in the library in step x

A(t,x) = 1e11 : Total number of molecules formed in PCR amplification in step x

D(t,x) = 1e8 : Diversity (number of different molecules in library) in step x

D(a,1) = 1e8, D(b,1) = 16, D(c,1) = 8, D(d,1) = 4, D(e,1) = 2, D (f,1) = 1

R(a) = 1e-7, R(b) = 6,25e-3, R(c) = 1,25e-2, R(d) = 2,5e-2, R(e) = 5e-2, R (f) = 1e-1

SO(x) = 0,8 : Fraction of homoduplexes surviving MISE treatment

SE(x) = 1e-4 : Fraction of heteroduplexes surviving MISE treatment

Table 1.1. Fraction of molecules in pool p in step x [N(p,x) divided by N(t,x)] after each amplification step. (Numbers in percent).

Step (x)		Pool (p)						
		a	b	c	d	e	f	Σ_{b-f}
1		99,999969	0,000016	0,000008	0,000004	0,000002	0,000001	0,000031
2		95,238095	0,952381	0,952381	0,952381	0,952381	0,952381	4,761905
3		63,670137	1,558650	2,656209	4,851337	9,241583	18,022084	36,329863
4		0,529398	0,050570	0,251337	1,591524	11,378731	86,198439	99,470602
5		0,000177	0,000019	0,000189	0,008976	0,867189	99,123449	99,999823
6		0,000000	0,000000	0,000000	0,000002	0,004046	99,995951	100,000000

As can be seen the library consisting of > 95 % background binders (pool a) after selection (step 2) consists of only 0,5 % background binders and 99 % binders (the sum of pools b-f [Σ_{b-f}]) after selection and 2 rounds of MISE (steps 3 and 4) using the described conditions.

Calculated example B (lower enrichment factor)

A model library composed of 5 pools (b-f) that contain species [pool b contains 16 species, pool c contains 8 species etc.] that bind target specifically and can be enriched from 0,01 fold (pool f) to 6,25e-4 fold (pool b). Pool a contains 1e8 species (minus the sum of the other pools) that bind unspecifically and therefore are depleted relatively from the library during selection: R(a) = 1e-7. In this case the selection step reduces the number of molecules in pool a from approximately 1e11 to 1e11*R(a) = 1e4 and the number of molecules in pool f from 1000 to 10.

N(t,x) = 1e11 : Total number of molecules in the library in step x

A(t,x) = 1e11 : Total number of molecules formed in PCR amplification in step x

D(t,x) = 1e8 : Diversity (number of different molecules in library) in step x

D(a,1) = 1e8, D(b,1) = 16, D(c,1) = 8, D(d,1) = 4, D(e,1) = 2, D (f,1) = 1

R(a) = 1e-7, R(b) = 6,25e-4, R(c) = 1,25e-3, R(d) = 2,5e-3, R(e) = 5e-3, R (f) = 1e-2

SO(x) = 0,8 : Fraction of homoduplexes surviving MISE treatment

SE(x) = 1e-4 : Fraction of heteroduplexes surviving MISE treatment

Table 1.2. Fraction of molecules in pool p in step x [N(p,x) divided by N(t,x)] after each amplification step. (Numbers in percent).

Step (x)	Pool (p)						
	a	b	c	d	e	f	Σ_{b-f}
1	99,999969	0,000016	0,000008	0,000004	0,000002	0,000001	0,000031
2	99,502488	0,099502	0,099502	0,099502	0,099502	0,099502	0,497512
3	99,088563	0,099085	0,106137	0,141386	0,211904	0,352926	0,911437
4	95,128061	0,095119	0,111690	0,234614	0,763023	3,667493	4,871939
5	19,132181	0,019130	0,025357	0,114356	1,807232	78,901744	80,867819
6	0,008267	0,000008	0,000011	0,000098	0,026939	99,964675	99,991733

As can be seen the library consisting of > 99,5 % background binders (pool a) after selection (step 2) consists of only 19 % background binders and > 80 % binders (the sum of pools b-f [Σ_{b-f}]) after selection and 3 rounds of MISE (steps 3,4 and 5) using the described conditions.

Calculated example C (Effect of more efficient mismatch cleavage)

A model library composed of 5 pools (b-f) that contain species [pool b contains 16 species, pool c contains 8 species etc.] that bind target specifically and can be enriched from 0,1 fold (pool f) to 6,25e-3 fold (pool b). Pool a contains 1e8 species (minus the sum of the other pools) that bind unspecifically and therefore are depleted relatively from the library during selection: R(a) = 1e-7. In this case the selection step reduces the number of molecules in pool a from approximately 1e11 to 1e11*R(a) = 1e4 and the number of molecules in pool f from 1000 to 100.

N(t,x) = 1e11 : Total number of molecules in the library in step x

A(t,x) = 1e11 : Total number of molecules formed in PCR amplification in step x

D(t,x) = 1e8 : Diversity (number of different molecules in library) in step x

D(a,1) = 1e8, D(b,1) = 16, D(c,1) = 8, D(d,1) = 4, D(e,1) = 2, D (f,1) = 1

R(a) = 1e-7, R(b) = 6,25e-3, R(c) = 1,25e-2, R(d) = 2,5e-2, R(e) = 5e-2, R (f) = 1e-1

SO(x) = 0,8 : Fraction of homoduplexes surviving MISE treatment

SE(x) = 1e-5 : Fraction of heteroduplexes surviving MISE treatment

Table 1.3. Fraction of molecules in pool p in step x [N(p,x) divided by N(t,x)] after each amplification step. (Numbers in percent).

Step (x)		Pool (p)						
		a	b	c	d	e	f	Σ_{b-f}
1		99,999969	0,000016	0,000008	0,000004	0,000002	0,000001	0,000031
2		95,238095	0,952381	0,952381	0,952381	0,952381	0,952381	4,761905
3		39,292097	2,026837	3,971975	7,862268	15,642835	31,203988	60,707903
4		0,022674	0,023500	0,177898	1,388845	10,985145	87,401938	99,977326
5		0,000001	0,000001	0,000057	0,006308	0,783933	99,209701	99,999999
6		0,000000	0,000000	0,000000	0,000000	0,003142	99,996858	100,000000

As can be seen the library consisting of > 95 % background binders (pool a) after selection (step 2) consists of only < 40 % background binders and > 60 % binders (the sum of pools b-f [Σ_{b-f}]) after selection and 1 round of MISE (step 3) using the described conditions.

These theoretical examples show that one can extract specific oligonucleotides from a diverse pool based on formation of homo- and hetero-duplexes. These examples also illustrate that parameters such as diversity, enrichment factor, library size and degree of separation of homo- and hetero-duplexes will influence the outcome of the mismatch selection treatment.

EXAMPLE 2. *General experimental procedure and material for single codon oligonucleotides*

Double stranded DNA species are formed by extension of oligonucleotide primer F on single stranded templates N_0 and N_{12} using Sequenase 2.0 (Amersham Biosciences) and buffer.

Heat-denaturation and annealing of samples:
A sample of either double stranded N_0 or double stranded N_{12} or a mixture of these in 5 µl of water containing 40 mM HEPES pH 7.5, 50 mM NaCl, 16 mM $MgCl_2$ was heated to 95°C for 10 minutes and allowed to anneal by lowering the temperature.

If only double stranded N_0 is present in the sample, this treatment is expected to result in the re-formation of homoduplexes.

If only double stranded N_{12} (4^12 = 1,7*10^7 different species) is present in the sample, this treatment is expected to result in the re-formation of homoduplexes and the formation of heteroduplexes.

If both double stranded N_0 and double stranded N_{12} is present in the sample, this treatment is expected to result in the re-formation of N_0 and N_{12} homoduplexes and the formation of N_{12}-N_{12} and N_0-N_{12} heteroduplexes (containing 1 or more mismatched base pairs).

Treatment of samples with mismatch-specific enzyme:
To a sample consisting of either double stranded N_0 or double stranded N_{12} or a mixture of these was added 1 µl (10x) Surveyor reaction buffer, 0.5 µl Enhancer, 1 µl Surveyor Nuclease, and 2.5 µl H2O. The samples were then mixed and incubated 1 hour at 42°C. Then the samples were heated to 95°C for 10 minutes and allowed to anneal by lowering the temperature. To 3 µl of each sample was added 1 µl 5xEXT buffer [100 mM HEPES pH 7.5, 750 mM

NaCl, 40 mM $MgCl_2$], 4 µl H_2O, and 1 µl E. coli Exonuclease VII (10 U/µl) (USB). The samples were then incubated at 37°C for 16 hours.

QPCR (quantitative real-time polymerase chain reaction) analyses to estimate number of molecules of each species before and after treatment. The QPCR was performed using a Taqman probe which is a fluorescence resonance energy transfer (FRET) probe consisting of a short oligonucleotide complementary to one of the amplified strands. The probe contains a fluorofor and a quencher molecule at the 5' and 3' end of the probe, respectively. This probe is included in the real-time PCR reaction along with the required forward and reverse PCR primers. The quencher molecule quenches the fluorescence of the fluorofor due to its close proximity on the probe. As the Taq polymerase replicates the new strand of the DNA, its 5'-3' exonuclease activity degrades the FRET probe from the 5'-end. This degradation releases the reporter flurofor from its proximity to the quencher, resulting in fluorescence of the reporter. Accumulation of fluorescence as a result of target amplification was detected in real time in an ABI 7900 HT sequence detection system (Applied Biosystems) which contains an optical detection systems During the exponential amplification phase, the amount of target should be doubling every cycle. Quantification analyses use the C_T value, which is the point (cycle number) at which the fluorescence signal reaches a specific threshold level of detection in the exponential phase. The more abundant the template, the earlier this point is reached. The quantity of DNA in the sample can be obtained by interpolation of its C_T value vs. a linear standard curve of C_T values obtained from a serially diluted standard solution.

Q-PCR reactions
For 5 ml premix (for one 96-well plate):
2.5 ml Taqman Universal PCR Master Mix (Applied Biosystems)
450 µl RPv2 (10 pmol/µl)
25 µl Taqman probe (50 µM)
1075 µl H_2O

40.5 µl premix was aliquoted into each well and 4.5 µl of relevant upstream PCR primer (Primer F for standard curve and N_{12}) or the N_0 specific primers QP1-3 and 5 µl sample (H_2O in wells for negative controls) was added.

The samples for the standard curve was prepared by diluting Temp4 to 10^8 copies/5 µl and subsequently performing a 10-fold serial dilution of this sample. 5 µl was used for each Q-PCR reaction.

Thermocycling/measurement of fluoresence was performed on an Applied Biosystems ABI Prism 7900HT real-time instrument utilizing the cycling parameters:
95°C 10 min
40 cycles of
95°C 15 sec
64°C 1 min

Oligonucleotides
N_{12} oligo:
5'-GTCAGAGACGTGGTGGAGGAAGTCTTCCTAGAAGCTGGA
NNNNNNNNNNNNTCTAGCAGCTAGTATGACGTGGTGTCCAAGCTG-3'

N_0 oligo:
5'-GCTAGAGACGTGGTGGAGGAAGTCTTCCTAGAAGCTGGA
TATCTTCAGTTCTCGACTCCTGAGTATGACGTGGTGTCCAAGCTG-3'

Primer F:
5'- CAGCTTGGACACCACGTCATAC -3'

Primer R:
5'- GTCAGAGACGTGGTGGAGGAA-3'

QP1-3:

5'-TCATACTCAGGAGTCGAGAACTGAAGATA-3'

Temp4:

5'-

GCTAGAGACGTGGTGGAGGAAGTCTTCCTAGAAGCTGGATATCT-GACGTGTTGAC

GTACACAGTATGACGTGGTGTCCAAGCTG-3'

TaqMan probe:

5'-6FAM-TCCAGCTTCTAGGAAGAC-MGB-NFQ (Applied Biosystems)

6FAM : 6-Carboxyfluorescein

MGB : Minor groove binder

NFQ : Non-fluorescent quencher

EXAMPLE 3: Discrimination between homo- and heteroduplexes in an identifier oligonucleotide containing a codon.

This example shows the possibility to specifically remove/degrade/separate heteroduplexes from homoduplexes. This is illustrates using a mismatch cleavage enzyme but other techniques such as for example gel separation, mismatch binding and column separation can also be used.

Two samples were subjected to the experimental procedure described in Example 2:

Sample 2A: 1 pmol double stranded N_0 in 5 µl of water containing 40 mM HEPES pH 7.5, 50 mM NaCl, 16 mM $MgCl_2$

Sample 2B: 1 pmol double stranded N_{12} in 5 µl of water containing 40 mM HEPES pH 7.5, 50 mM NaCl, 16 mM $MgCl_2$

Results of QPCR analyses of sample 2A:

Number of homoduplexes before MISE treatment: 6, 71E+08 (6, 00E+08 expected)

Number of homoduplexes after MISE treatment: 4, 54E+08
Thus (4, 54 / 6, 71) = 67 % of homoduplexes have survived the MISE treatment

Results of QPCR analyses of sample 2B:
Number of heteroduplexes before MISE treatment: 7, 35E+08
(6,00E+08expected)
Number of heteroduplexes after MISE treatment: 1, 16E+07
Thus (1, 16 / 73, 5) = 1, 5 % of homoduplexes have survived the MISE treatment

That is the relative survival factor of homoduplexes is (67 / 1, 5) = 45

The relative survival factor obtain in this example is dependent on the experimental conditions. This can be future optimized if required by tuning the mismatch treatment conditions. The relative survival factor could also be different using other techniques in specifically remove/degrade/separate heteroduplexes from homoduplexes, as for example gel separation, mismatch binding and column separation.

EXAMPLE 4: Enrichment of sequences in excess over a background sequence population.

This example describes the possibility to enrich a specific oligonucleotide among a diverse background of sequences.

Samples were subjected to the experimental procedure described in Example 2:

A mixed sample containing 1 pmol sample N_{12} (4^12 = 1,7*10^7 different species of double stranded DNA) and 0,001 pmol of sample N_0 in 5 µl of water containing 40 mM HEPES pH 7.5, 50 mM NaCl, 16 mM $MgCl_2$ was subjected to general procedure 2. Then samples were diluted 300 times and analyzed by QPCR (input) and the result from one mismatch selection treatment (output) as described in Example 2

QPCR analysis result:

Sample	# N_{12}	# N_0	Fold excess N_{12}
Input	5,11E+08	1,27E+05	4040
Output	3,80E+03	6,58E+01	58

The enrichment factor with the mismatch selection procedure is 70 (4040/58) in this experiment.

This example demonstrates the possibility to enrich for a specific oligonucleotide sequence (N_0) among a diverse pool of oligonucleotide sequences (N_{12}). The specific sequence (N_0) is in 17000-fold excess over each specific N_{12} sequences but in 1000-fold (4040 experimentally) lower concentration compared to the entire population of N_{12} sequences. Thus, although the background sequences are in excess, the specific sequence is able to survive the mismatch selection treatment.

EXAMPLE 5: Sequential mismatch selection treatment

One important feature with mismatch selection is that one can perform multiple round of treatment. This will permit extreme enrichment factors which might be important when the library size is much larger than the enrichment factor obtain in the initial selection.

A mixed sample containing 1 pmol sample N_{12} (4^12 = $1,7*10^7$ different species of double stranded DNA) and 0,0001 pmol of sample N_0 in 5 µl of water containing 40 mM HEPES pH 7.5, 50 mM NaCl, 16 mM $MgCl_2$ was subjected to condition described in Example 2.

Following the procedure described in Example 2, the samples were run on a denaturing 10 % polyacrylamide gel and a gel slice bands containing full-length templates (N_0 or N_{12}) [estimated using 32P-labeled marker oligos of the same length as N_0 and N_{12} were excised from the gel and placed in an eppendorph tube. 100 µl of 1 x EXT buffer [40 mM HEPES pH 7.5, 50 mM

NaCl, 16 mM $MgCl_2$] was added. DNA was liberated from the gel by freeze-thawing using 2 cycles of heating to 99°C and cooling to -20°C. 400 µl H_2O was added and 5 µl DNA mixture was used for PCR amplification with 5 pmol each of primers F and R in a 25 µl reaction using Ready-To-Go beads (Amersham Biosciences).

Approximately 1 pmol of amplified material (containing N_0 and N_{12}) was subjected to another round of mismatch selection as described in Example 2, except that the Exo VII treatment step was excluded. Then the sample was diluted 300 times and analyzed by QPCR

QPCR analysis result:

Sample	# N_{12}	# N_0	Fold excess N_{12}
Input	1,54E+09	3,42E+04	45098
Output 1st round of general procedure 2	1,30E+04	1,99E+02	66
Output 2nd round of general procedure 2	3,53E+03	7,28E+02	5

The enrichment factor in the 1st round of mismatch selection treatment described in Example 2 is 45098 / 66 = 683

The enrichment factor in the 2nd round of mismatch selection treatment described in Example 2 (excluding the Exo VII treatment) is 66 / 5 = 13

The total enrichment factor from these two mismatch selection treatment is 8879 (683*13).

This example shows two rounds of mismatch selection treatment but this treatment can be continued until desired result is obtained.

This enrichment factor obtained with the mismatch selection treatment can then be multiplied with the initial enrichment factor obtained with the standard selection procedure to obtain the overall enrichment factor for the complete selection process.

EXAMPLE 6. *General experimental procedure and material for multiple codon oligonucleotides*

Double stranded DNA species are formed by extension of oligonucleotide primer ER on single stranded templates EN_0, EN_6 and EN_{12} using Sequenase 2.0 (Amersham Biosciences) and buffer.

Heat-denaturation and annealing of samples:
A mixture of double stranded EN_0, double stranded EN_6 and double stranded EN_{12} in 5 µl of water containing 40 mM HEPES pH 7.5, 50 mM NaCl, 16 mM $MgCl_2$ was heated to 95°C for 10 minutes and allowed to anneal by lowering the temperature.

This treatment is expected to result in the re-formation of EN_0-EN_0, EN_6-EN_6, and EN_{12}-EN_{12} homoduplexes and the formation of EN_0-EN_6, EN_0-EN_{12}, EN_6-EN_6, EN_6-EN_{12} and EN_{12}-EN_{12} heteroduplexes (containing 1 or more mis-matched base pairs).

Treatment of samples with mismatch-specific enzyme:
To the 5 µl mixture was added 1 µl (10x) Surveyor reaction buffer, 0.5 µl Enhancer, 1 µl Surveyor Nuclease, and 2.5 µl H2O. The samples were then mixed and incubated 1 hour at 42°C. Then the sample were heated to 95°C for 10 minutes and allowed to anneal by lowering the temperature. To 3 µl of each sample was added 1 µl 5xEXT buffer [100 mM HEPES pH 7.5, 750 mM NaCl, 40 mM $MgCl_2$], 4 µl H_2O, and 1 µl E. coli Exonuclease VII (10 U/µl) (USB). The samples were then incubated at 37°C for 16 hours.

Then the samples were run on a denaturing 10 % polyacrylamide gel and a gel slice bands containing full-length templates (EN_0, EN_6 and EN_{12}) [estimated using 32P-labeled marker oligos of the same length as the templates) were excised from the gel and placed in an eppendorph tube. 100 µl of 1 x EXT buffer [40 mM HEPES pH 7.5, 50 mM NaCl, 16 mM $MgCl_2$] was added.

DNA was liberated from the gel by freeze-thawing using 2 cycles of heating to 99°C and cooling to -20°C. 400 µl H_2O was added and 5 µl DNA mixture was used for PCR amplification with 5 pmol each of primers EF and ER in a 25 µl reaction using Ready-To-Go beads (Amersham Biosciences).

A TOPO-TA (Invitrogen) ligation reaction was assembled with 4 µl PCR product, 1 µl salt solution (Invitrogen), and 1 µl vector. Water was added to 6 µl. The reaction was incubated at RT for 30 min. Heat-shock competent TOP10 E.coli cells were thawed on ice. 5 µl ligation reaction was added to the thawed cells and these were incubated 30 min on ice, heatshocked in 42°C water for 30 sec, and then put on ice. 250 µl of growth medium was added and the mixture was incubated 1 h at 37oC. The mixture was then spread on a growth plate containing 100 µg / ml ampicillin and incubate ON at 37oC. Individual *E.coli* clones were picked and transferred to PCR wells containing 50 µl water. These 50 µl were incubated at 94°C for 5 minutes and used in a 20 µl in a 25 µl PCR reaction with 5 pmol of each TOPO primer M13 forward & M13 reverse and Ready-To-Go PCR beads (Amersham Biosciences). The following PCR profile was used: 94oC 2 min, then 30 x (94oC 4 sec, 50oC 30 sec, 72oC 1 min) then 72oC 10 min. Primers and nucleotides were degraded by adding 1 µl 1:1 EXO/SAP mixture (USB corp.) to 2 µl PCR product and incubating at 37°C for 15 min and then 80°C for 15 min to heat-inactivate the enzymes. 5 pmol T7 primer was added and water was added to 12 µl. Add 8 µl DYEnamic ET cycle sequencing Terminator Mix (Applied biosystems). A thermocycling profile of 30 x (95oC 20 sec, 50oC 15 sec, 60oC 1 min) was run. Then 5 µl water was added to each well and sequencing reactions were purified using seq96 spinplates (Amersham Biosciences). Reactions were run on a MegaBace capillary electrophoresis instrument (Molecular Dynamics) using injection parameters 2 kV, 50 sec and run parameters: 9 kV 45 min and analyzed using Contig Express software (Informax).

Oligonucleotides

EN_0 oligo:
5'GTCGAATGCTGTAGCGGTAGGCAGCAATGACGTCGAATGACAGCAAA
TGAGTCGATGTGCTGAGCTAGAT-3'

EN_6 oligo:
5'- GTCGAATGCTGTAGCGGTAGGCAGCANNGACGTCGNANGACAGCA
NATNAGTC
GATGTGCTGAGCTAGAT -3'

EN_{12} oligo:
5'-
GTCGAATGCTGTAGCGGTAGGCAGCNNTNNCGTCGANNNNCAGCANN-
NGNGTC
GATGTGCTGAGCTAGAT-3'

EF oligo:
5'-GTCGAATGCTGTAGCGGTAG

ER oligo:
5'-ATCTAGCTCAGCACATCGAC

M13 forward:
5'-GTAAAACGACGGCCAG
M13 reverse:
5'-CAGGAAACAGCTATGAC
T7 primer:
5'-TAATACGACTCACTATAGGG

EXAMPLE 7: Mismatch selection treatment and composition of oligonucleotide containing multiple codons.

Libraries of bifunctional complexes will preferably contain more than one codon allowing encoding of multiple functional entities in the displayed molecule. This example describes an identifier containing three variable regions that represent three individual encoding codons. In the homo- hetero-duplex separation step in the mismatch cleavage procedure multiple short fragments are produced that could potentially recombine through overlapping sequences and generated shuffled variants. These shuffled identifiers would then contain codons originated from different original identifiers. This is tested in this example.

Selected libraries with identifier oligos containing 3 codons was modelled using templates EN_0, EN_6, and EN_{12}.
It is expected that a library initially containing 4^12 = 1,6e7 different species will contain an enriched best binder EN_0 and a pool of not-best binders EN_6. The enriched pool of not-best is expected to be diverse (in this case the EN_6 pool contains 4^6 = 4096 species but not as diverse as the background of non-specific binders (EN_{12} contains 1,6e7 different species).

Two samples were mixed to model selected libraries:

Sample 3.1A
1 pmol EN_{12}, 0.1 pmol EN_6, 0.1 pmol EN_0. This corresponds to a 1,6e7 member library that has been selected so that the best binder (EN_0) has been enriched 1,6e6 fold and the not-best binders (EN_6) have been enriched 390 fold (1,6e6 / 4096).

Sample 3.1B
1 pmol EN_{12}, 0.01 pmol EN_6, 0.01 pmol EN_0. This corresponds to a 1,6e7 member library that has been selected so that the best binder (EN_0) has been enriched 1,6e5 fold and the not-best binders (EN_6) have been enriched 39 fold (1,6e5 / 4096).

The samples were subjected mismatch selection as described in Example 6.

Sample 3.1A

	EN_{12}	EN_6	EN_0
Input (sequencing)	11	2	2
Output (sequencing)	5	0	6

This corresponds to an EN_0 enrichment fold of (6/11)/(2/(2+2+11)) = 4

INPUT 1 N_0 : 1 N_6 : 10 N_{12}

```
» F01    (1)  GCTGTAGCGGTAGGCAGCAATCACGTCGAATCACAGCAAATCAGTCGATGTGCTGAGCTAG
» B02    (1)  GCTGTAGCGGTAGGCAGCAATCACGTCGAATCACAGCTAATCAGTCGATGTGCTGAGCTAG
» G02   (32)  GCTGTAGCGGTAGGCAGCAGTCACGTCGAAACACAGCAAATCAGTCGATGTGCTGAGCTAG
» D01   (32)  GCTGTAGCGGTAGTCAGCACGCACGTCGAAACACAGCAGATCAGTCGATGTGCTGAGCTAG
» E03   (32)  GCTGTAGCGGTAGGCAGCTATACCGTCGATCCACAGCAGGAGGGTCGATGTGCTGAGCTAG
» E01   (29)  GCTGTAGCGGTAGGCAGCCATAACGTCGACGACCAGCAGCAGAGTCGATGTGCTGAGCTAG
» E02   (32)  GCTGTAGCGGTAGGCAGCTTTTCCGTCGATGGTCAGCAGCAGGGTCGATGTGCTGAGCTAG
» F02   (32)  GCTGTAGCGGTAGGCAGCGGTCTCGTCGATGTACAGCAAAAGAGTCGATGTGCTGAGCTAG
» C01   (53)  GCTGTAGCGGTAGTGCAGCCGTACGTCGAGNCAGCAGCACAGAGTCGATGTGCTGAGCTAG
» F03   (32)  GCTGTAGCGGTAGGCAGCCATTGCTTCGAGCCACAGCAAGGGGGTCGATGTGCTGAGCTAG
» A03   (32)  GCTGTAGCGGTAGGCAGCAATAGCGTCGAGGGACAGCAGCCGGGTCGATGTGCTGAGCTAG
» H03   (32)  GCTGTAGCGGTAGGCAGCGGTACCGTCGAATCACAGCACCTGAGTCGATGTGCTGAGCTAG
» A02   (32)  GCTGTAGCGGTAGGCAGCGGTAGCGTCGAAGGACAGCAGATGAGTCGATGTGCTGAGCTAG
» D03   (32)  GCTGTAGCGGTAGGCATCCGTAGCGTCGAAAGTCAGCAGGGGAGTCGATGTGCTGAGCTAG
» C04   (32)  GCTGTAGCGGTAGGCAGCGGTATCGTCGAAAGACAGCAGCAGGGTCGATGTGCTGAGCTAG
```

⇩ 1r MISE

OUTPUT 1 N_0 : 1 N_6 : 10 N_{12}

```
» A05    (33)  GCTGTAGCGGTAGGCAGCAATCACGTCGAAACACAGCATTTCAGTCGATGTGCTGAGCTAG
» B05 (2) (33)  GCTGTAGCGGTAGGCAGCAATCACGTCGAATCACA-CAAATCAGTCGATGTGCTGAGCTAG
» D05    (33)  GCTGTAGCGGTAGGCAGCAATCACGTCGAATCACAGCAAATCAGTCGATGTGCTGAGCTAG
» A06 (2) (35)  GCTGTAGCGGTAGGCAGCAATCACGTCGAATCACAGCAAATCAGTCGATGTGCTGAGCTAG
» A06    (33)  GCTGTAGCGGTGGGCAGCAATCACGTCGAACCACAGCAAATAAGTCGATGTGCTGAGCTAG
» C05    (29)  GCTGTAGCGGTAGGCAGCAATCACGTCGAATCTCAACAAATCAGTCGATGTGCTGAGCTAG
» B06          GCTGTAGCGGTAGGCAGCGATTACGTCGACAGTCAGCACAAGGGTCGATGTGCTGAGCTAG
» E06    (34)  GCTGTAGCGGTAGGCAGCAGTCTCGTCGATGGCCAGCAGGAGAGTCGATGTGCTGAGCTAG
» G06 (2) (34)  GCTGTAGCGGTAGGGAGCCTTAACGTCGAACGCCAGCAGAAGCGTCGATGTGCTGAGCTAG
» C05 (2) (28)  GCTGTAGCGGTAGGCAGCAATA-CGTCGATATGCAGCAAGAGCGTCGATGTGCTGAGCTAG
» H06 (2) (34)  GCTGTAGCGGTAGGCAGCAGTACCGTTGAACAACAGCAGGCGAGTCGATGTGCTGAGCTAG
```

Sample 3.1B

	EN_{12}	EN_6	EN_0
Input (theoretical)	100	1	1
Output (sequencing)	6	3	1

This corresponds to an EN_0 enrichment fold of (1/(6+3+1))/(1/100) = 10

and an EN_6 enrichment fold of (3/(6+3+1))/(1/100) = 30

INPUT 1 N_0 : 1 N_6 : <u>100</u> N_{12}

1r MISE

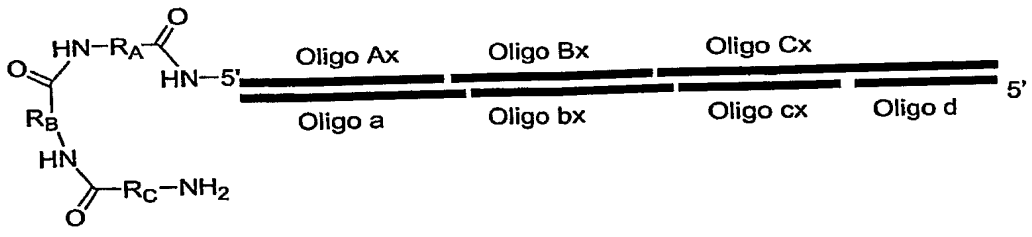
OUTPUT 1 N_0 : 1 N_6 : <u>100</u> N_{12}

```
» F03   (31)  GCTGTAGCGGTAGGCAGCAAAGACGTCGAATGACAGCAGATGAGTCGATGTGCTGAGCTAG
» G03   (29)  GCTGTAGCGGTAGGCAGCAGTGACGTCGAAGACAGCAAATAAGTCGATGTGCTGAGCTAG
» A03   (30)  GCTGTAGCGGTAGGCAGCAAAGACGTCGAAGACAGCAAATAAGTCGATGTGCTGAGCTAG
» C04    (1)  GCTGTAGCGGTAGGCAGCAGAGACGTCGCATGACAGCAGATAAGTCGATGTGCTGAGCTAG
» H02   (29)  GCTGTAGCGGTAGGCAGCAATAGCGTCGAGTACCAGCAGCTGAGTCGATGTGCTGAGCTAG
» E01   (30)  GCTGTAGCGGTAGGCAGCGATGACGTCGAGGGGCAGCAAAGGAGTCGATGTGCTGAGCTAG
» D02    (1)  GCTGTAGCGGTAGGCAGCAGTCACGTCGAGAAGCAGCAGGAGAGTCGATGTGCTGAGCTAG
» G01   (29)  GCTGTAGCGGTAGGCAGCGGTGACGTCGAAGTGCAGTAATAGGGTCGATGTGCTGAGCTAG
» A02   (25)  GCTGTAGCGGTAGGCAGTAATGCCGTCGA.CGACAGCACGGGAGTCGATGTGCTGAGCTAG
» D04   (30)  GCTGTAGCGGTAGGCAGCCTTAGCGTCGAATTGCAGCAGACGCGTCGATGTGCTGAGCTAG
```

These two results shows that enrichment of the expected sequences (N_0, N_6) are identified by sequencing and the composition of the identifier sequences are kept intact. There is no shuffling between the codons from the N_0, N_6, N_{12} oligonucleotides.

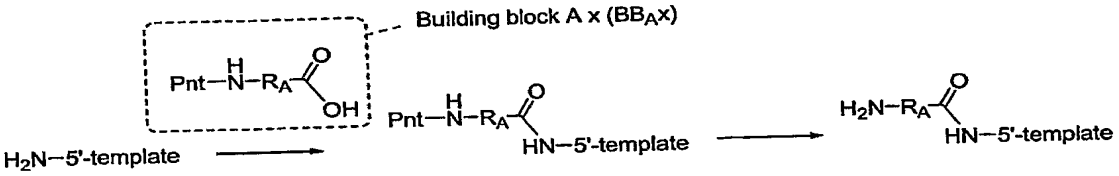
<u>Example 8 : Identification of a ligand from a library composed of 61875 different small molecules each associated with a unique identifer oligonucleotide by selection and subsequently using mismatch selection (MISE).</u>

General arrangement of each complex composed of display molecule and identifier oligonucleotide in the library generation:



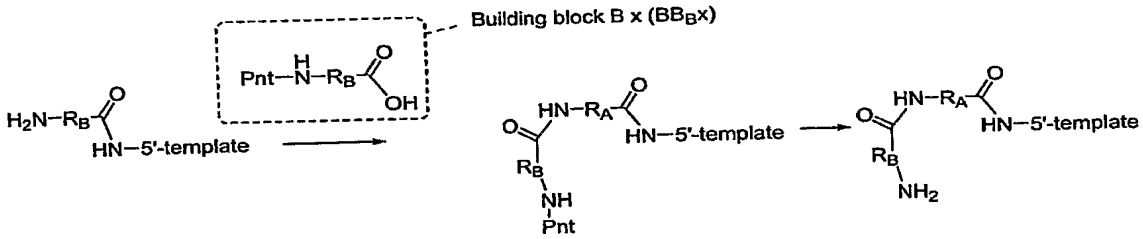
Overview of the library generation procedure:

First round of library generation (round A) :

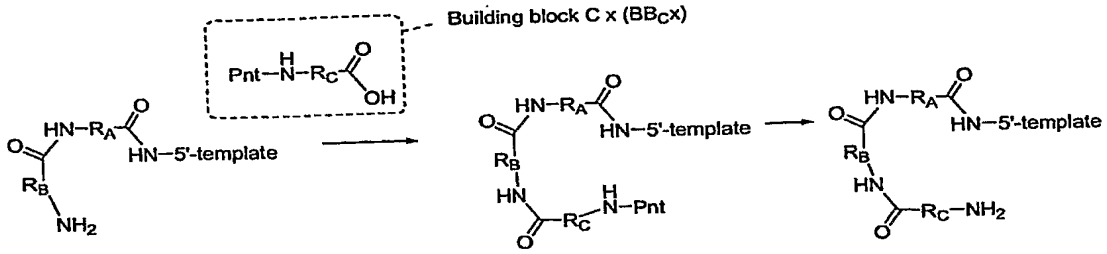


"Pnt" corresponds to pentenoyl – an amine protecting group. "R" can by any molecule fragment. The chemical used in library generation comprise a primary (shown) or a secondary amine.

Second round of library generation (round B) :



Third round of library generation (round C):



General procedure : Library generation, selection and mismatch subsequent selection

First round of library generation (round A) :

First oligonucleotides of the A series are each modified by adding to each type of oligo a small molecule building block (BB_AX) to the 5' amine forming an amide bond. After this step the template is comprised of oligo Ax.

Second round of library generation (round B) :

4 nmol of a mixture of different modified A oligos are then split into a number tubes corresponding to the number of different building blocks to be used in round B. 190 pmol Oligo a and 2 µl heering DNA is added to each tube and the DNA material in each tube is lyophilized. The lyophilized DNA is then re-dissolved in 50 µl water and purified by spining through Biospin P-6 columns (Biorad) equilibrated with water.

Addition of building block:

The DNA material in each tube is again lyophilized and redissolved in 2 µl 100 mM Na-borate pH 8.0/100 mM sulfo N-hydroxy succinimide (sNHS). For each tube 10 µl building block BB_BX (100 mM in dimethyl sulfoxide [DMSO]) is preactivated by mixing with 10 µl 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (90 mM in dimethylformamide [DMF]) and incubating at 30°C for 30 min. 3 µl of this preactivated mixture is then mixed with the 2 µl in each tube and allowed to react 45 min at 30 °C. Then an additional 3 µl freshly preactivated BB is added and the reaction is allowed to proceed for

45 min at 30 °C. The resulting mixture is then purified by spinning through Bio-Rad P6 DG (Desalting gel).

Addition of codon oligonucleotide :

The DNA material is then lyophilized and redissolved in 10 µl water containing 200 pmol oligo Bx (eg. B1) and the corresponding oligo bx (eg. b1). This is done so that the codon in oligo Bx identifies the BB_Bx added to the DNA template. 10 units of T4 DNA ligase (Promega) and 1.2 µl T4 DNA ligase buffer is then added to each tube and the mixture is incubated at 20°C for 1 hour. The DNA template linked to the small molecules now comprises an Ax oligo with a Bx oligo ligated to its 3' end. The reactions are then pooled, an appropiate volume of water is allowed to evaporate and the remaining sample is purified by spining through Biospin P-6 columns (Biorad) equilibrated with water.

Removal of building block protecting group:

The pooled sample (~ 50 µl) is adjusted to 10 mM Na-acetate (pH 5). 0.25 volumes of 25 mM Iodine in tetrahydrofuran/water (1:1) is added and the sample is incubate at 37 °C for 2h. The reaction is then quenched by addition of 2 µl of 1M $Na_2S_2O_3$ and incubation at room temperature for 5 min. The complexes are then purified by spining through Biospin P-6 columns (Biorad) equilibrated with water

To remove sulphonamide protecting groups, the sample is adjusted to 50 µl 100 mM sodium borate pH 8.5 and 20 µl 500 mM 4-methoxy thiophenol (in acetonitrile) is added and the reaction is incubated at 25°C overnight. Then the complexes are purified by spinning through Biospin P-6 columns (Biorad) equilibrated with water and then lyophilized.

Third round of library generation (round C) :

The samples are dissolved in 175 µl 100 mM Na-borate pH 8.0 and distributed into 25 wells (7 µl / well). 2 µl 100 mM BB_Cx in water/DMSO and 1 µl of

250 mM DMT-MM is added to each reaction and incubated at 30 °C overnigth. Water is added to 50 µl and the reactions are then spin purified using Bio-Rad P6 DG (Desalting gel) and subsequently water is allowed to evaporate so that the final volume is 10 µl.

Addition of building block:

The DNA material is then lyophilized and redissolved in 10 µl water containing 200 pmol oligo Cx (eg. C1) and the corresponding oligo cx (eg. c1). This is done so that the codon in oligo Cx corresponds to the BB_Cx added to the DNA template. 10 units of T4 DNA ligase (Promega) and 1.2 µl T4 DNA ligase buffer is then added to each tube and incubated at 20°C for 1 hour. The DNA template linked to the small molecules now comprises and Ax oligo with a Bx ligated to its 3' end and a Cx oligo ligated to the 3' end of the Bx oligo. The reactions are then pooled, the pooled sample volume is reduced by evaporation and the sample is purified by spining through Biospin P-6 columns (Biorad) equilibrated with water. The pooled sample (~ 50 µl) is adjusted to 10 mM Na-acetate (pH 5). 0.25 volumes of 25 mM Iodine in tetrahydrofuran/water (1:1) is added and the sample is incubate at 37 °C for 2h. The reaction is then quenched by addition of 2 µl of 1M $Na_2S_2O_3$ and incubation at RT for 5 min. Then the DNA templates (carrying small molecules) are purified by spinning through Biospin P-6 columns (Biorad) equilibrated with water and then lyophilized.

Final deprotection step

Some building blocks contain methyl esters that are deprotected to acids by dissolving the pooled sample in 5 µl 20 mM NaOH, heating to 80 °C for 10 minutes and adding 5 µl of 20 mM HCl.

Final extension step

To ensure that the DNA templates are double stranded prior to selection oligo d is extended along the template by adding to the sample 10 µl of 5 X sequenase EX-buffer [100 mM Hepes, pH 7.5, 50 mM $MgCl_2$, 750 mM NaCl]

and 4000 pmol oligo d. Annealing is performed by heating to 80°C and cooling to 20 °C. To the sample is then added 500 µL dNTP , water to 50 µl and 39 units of Sequenase version 2.0 (USB). The reaction is incubated at 37°C for 1 hour.

Selection

This library is subjected to selection, whereby binders to the selection target are enriched.

Maxisorp ELISA wells (NUNC A/S, Denmark) were coated with each 100 µL 2µg/mL integrin αVβ3 in PBS buffer [2.8 mM NaH_2PO_4, 7.2 mM Na_2HPO_4, 0.15 M NaCl, pH 7.2] overnight at 4°C. Then the integrin solution was substituted for 200 µl blocking buffer [TBS, 0.05% Tween 20 (Sigma P-9416), 1% bovine serum alnumin (Sigma A-7030), 1 mM $MnCl_2$] which was left on for 3 hours at room temperature. Then the wells were washed 10 times with blocking buffer and the encoded library was added to the wells after diluting it 100 times with blocking buffer. Following 2 hours incubation at room temperature the wells were washed 10 times with blocking buffer. After the final wash the wells were cleared of wash buffer and subsequently inverted and exposed to UV light at 300-350 nm for 30 seconds using a trans-illuminator set at 70% power. Then 100 µl blocking buffer without Tween-20 was immediately added to each well, the wells were shaken for 30 seconds, and the solutions containing eluted templates were removed for PCR amplification and then used for mismatch selection (MISE).

Mismatch selection (MISE) :

The double stranded sample is denatured by heating and allowed to cool whereby hetero- and homoduplexes are formed:

Treatment of samples with nucleases:

To the 5 µl mixture ia added 1 µl (10x) Surveyor reaction buffer (Transgenomic), 0.5 µl Enhancer (Transgenomic), 1 µl Surveyor Nuclease (Transgenomic), and 2.5 µl H_2O. The samples are then mixed and incubated 1 hour at

42°C. Then the sample is heated to 95°C for 10 minutes and duplexes are allowed to form by lowering the temperature. To 3 µl of each sample is added 1 µl 5 x EXT buffer [100 mM HEPES pH 7.5, 750 mM NaCl, 40 mM $MgCl_2$], 4 µl H_2O, and 1 µl E. coli Exonuclease VII (10 U/µl) (USB). The samples are then incubated at 37°C for 16 hours.

Purification by polyacrylamide gel electrophoresis:

Then the samples are mixed with loading buffer and run on a denaturing 10 % polyacrylamide gel and gel slices containing full-length templates (estimated using 32P-labeled marker oligos of the same length as the templates) are excised from the gel and placed in an eppendorph tube. 100 µl of 1 x EXT buffer [40 mM HEPES pH 7.5, 50 mM NaCl, 16 mM $MgCl_2$] are added. DNA is liberated from the gel by freeze-thawing using 2 cycles of heating to 99°C and cooling to -20°C. 400 µl H_2O is added and 5 µl DNA mixture is used for PCR amplification with 5 pmol each of forward and reverse primers and in a 25 µl reaction using Ready-To-Go beads (Amersham Biosciences).

Cloning of MISE products

A TOPO-TA (Invitrogen) ligation reaction is assembled with 4 µl PCR product, 1 µl salt solution (Invitrogen) and 1 µl vector. Water is added to 6 µl. The reaction is then incubated at RT for 30 min. Heat-shock competent TOP10 E.coli cells are then thawed on ice and 5 µl of the ligation reaction is added to the thawed cells. The cells are then incubated 30 min on ice, heatshocked in 42°C water for 30 sec, and then put on ice again. 250 µl of growth medium is added to the cells and they are incubated 1 h at 37°C. The medium containting cells is then spread on a growth plate containing 100 µg / ml ampicillin and incubated at 37°C for 16 hours.

Sequencing of MISE products:

Individual *E.coli* clones are then picked and transferred to PCR wells containing 50 µl water. These 50 µl were incubated at 94°C for 5 minutes and used in a 20 µl in a 25 µl PCR reaction with 5 pmol of each TOPO primer M13 for-

ward & M13 reverse and Ready-To-Go PCR beads (Amersham Biosciences). The following PCR profile is used: 94°C 2 min, then 30 x (94°C 4 sec, 50°C 30 sec, 72°C 1 min) then 72°C 10 min. Primers and nucleotides are then degraded by adding 1 µl 1:1 EXO/SAP mixture (USB corp.) to 2 µl PCR product and incubating at 37°C for 15 min and then 80°C for 15 min to heat-inactivate the enzymes. 5 pmol T7 primer is added and water is added to 12 µl. Then 8 µl DYEnamic ET cycle sequencing Terminator Mix (Applied biosystems) is added to each well. A thermocycling profile of 30 x (95°C 20 sec, 50°C 15 sec, 60°C 1 min) is then run. Then 10 µl water is added to each well and sequencing reactions are purified using seq96 spinplates (Amersham Biosciences). Reactions are then run on a MegaBace capillary electrophoresis instrument (Molecular Dynamics) using injection parameters 2 kV, 50 sec and run parameters: 9 kV 45 min and analyzed using Contig Express software (Informax).

Example 8.1 (general procedure 8): Generation of a library containing 61875 different small molecules and identification of a binder among these by subjecting the library to target selection and subsequent mismatch selection (MISE) :

First round of library generation (round A) :

99 different A oligos were used :

Oligo Ax general structure :

5'- **NSP**-ACCTCAGCTGTGTATCGAGCGGCAGCTGTTCCGTCG-3'

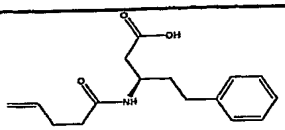
The underlined part corresponds to the 5 nucleotide sequence that varies among different A oligos, ie. the codon. The remaining sequence is identical among the A oligos.

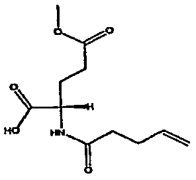
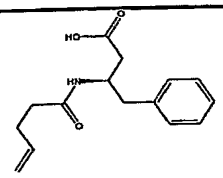
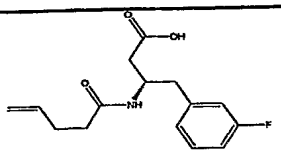
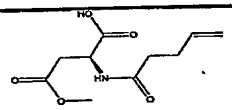
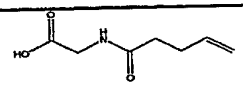
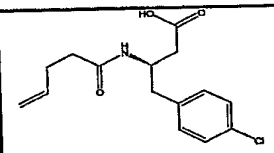
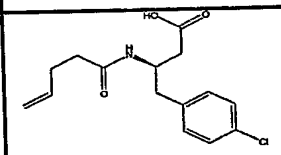
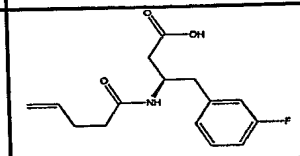
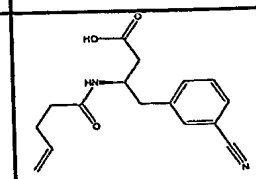
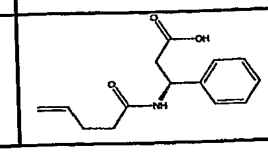
N : 5'-Amino-Modifier 5 (Glen research cat# 10-1905-90)
S : Spacer C3 CPG (Glen research cat# 20-2913-01)
P : PC Spacer Phosphoramidite (Glen research cat# 10-4913-90)

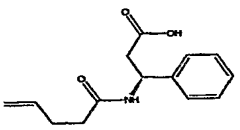
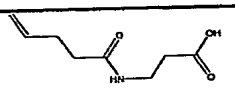
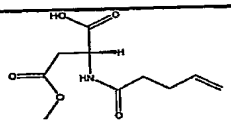
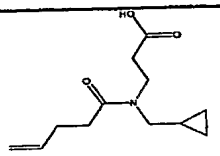
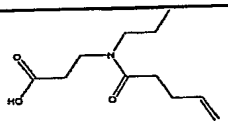
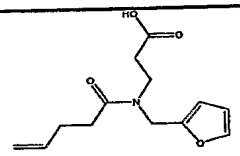
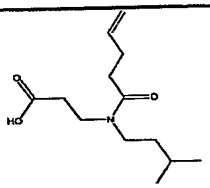
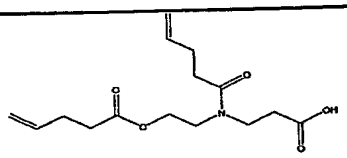
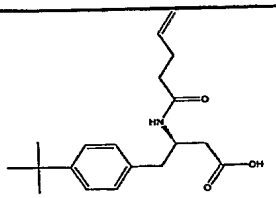
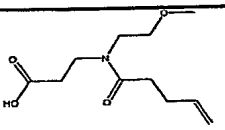
Oligo a: 5'- TGTGCGACGIIIIIGCTGCCGCTCGATACACAGCTGAGGT

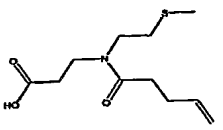
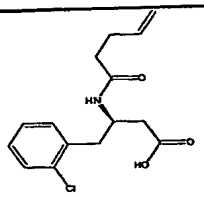
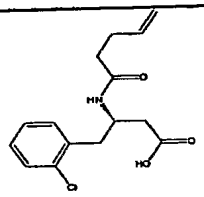
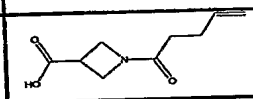
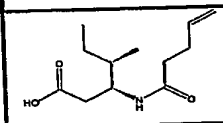
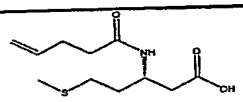
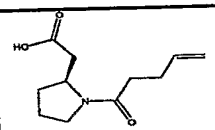
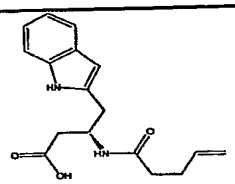
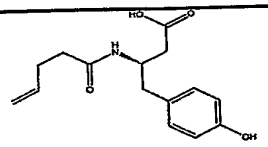
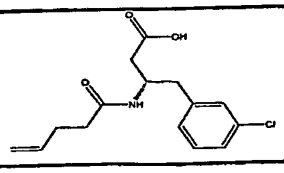
I : inosine

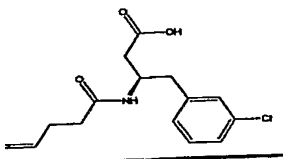
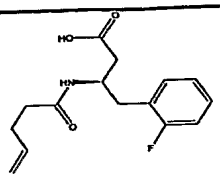
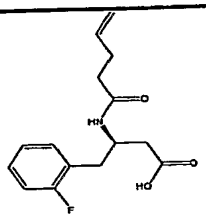
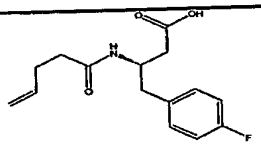
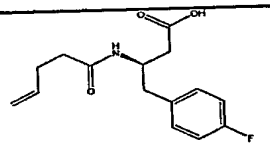
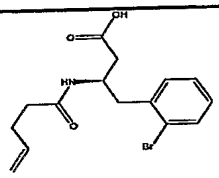
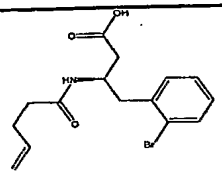
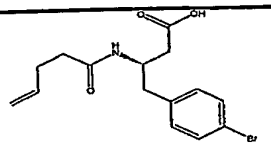
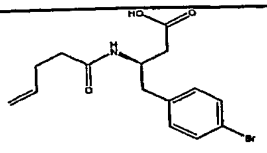
Onto the free NH_2 of the 5' amino modifier on these oligos were loaded the listed BB_Ax building blocks:

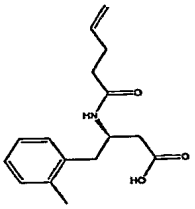
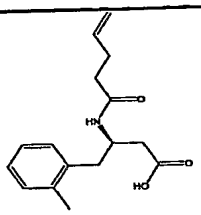
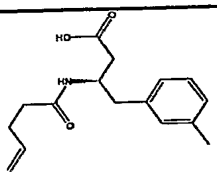
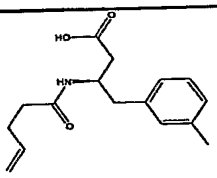
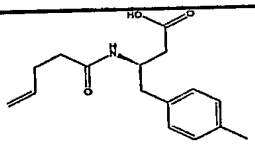
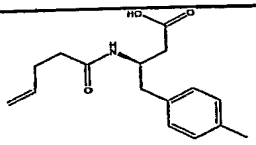
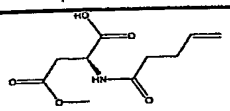
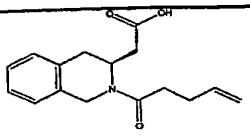
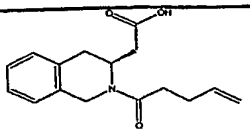
Oligo	Codon	Building block	Building block structure
Oligo A1	TGTTC	BB_A1	

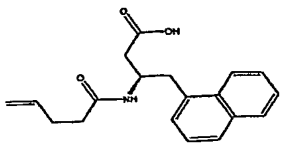
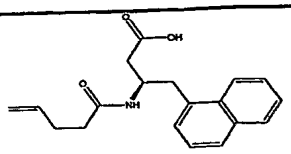
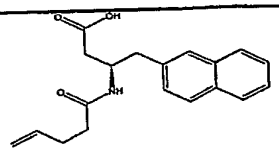
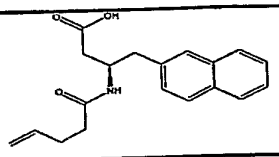
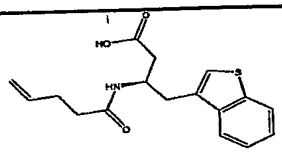
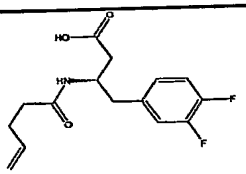
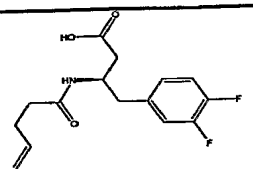
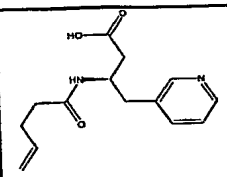
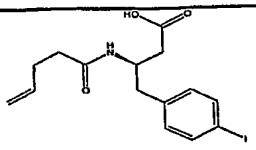
Oligo A2	CGAGC	BB_A2	
Oligo A3	GGATA	BB_A3	
Oligo A4	CGCTG	BB_A4	
Oligo A5	GTTAT	BB_A5	
Oligo A6	AGTGC	BB_A6	
Oligo A7	ACCTG	BB_A7	
Oligo A8	CTGGT	BB_A8	
Oligo A9	TAGGA	BB_A9	
Oligo A10	ACTCA	BB_A10	
Oligo A11	CTTAC	BB_A11	

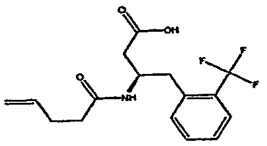
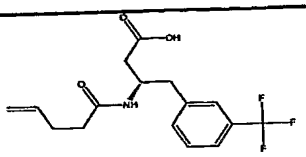
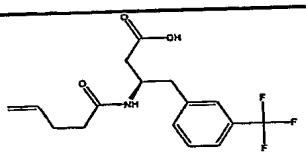
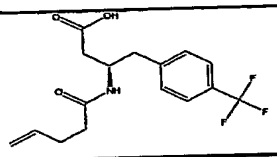
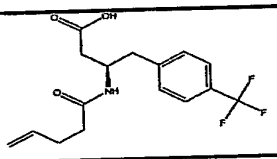
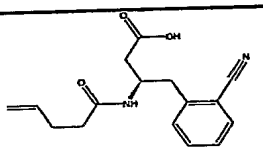
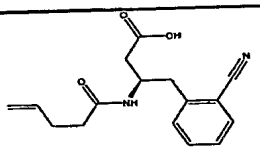
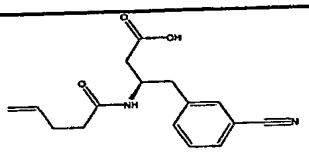
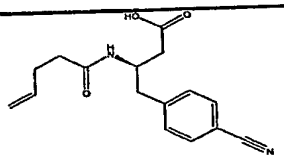
Oligo A12	CGCAC	BB_A12	
Oligo A13	TCGCG	BB_A13	
Oligo A14	CGGAT	BB_A14	
Oligo A15	GAGAT	BB_A15	
Oligo A16	TGTAG	BB_A16	
Oligo A17	GTGTT	BB_A17	
Oligo A18	AGATG	BB_A18	
Oligo A19	ATCCT	BB_A19	
Oligo A20	TTGCT	BB_A20	
Oligo A21	ACGTA	BB_A21	

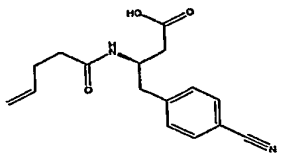
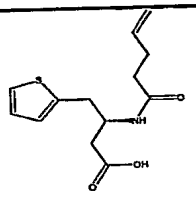
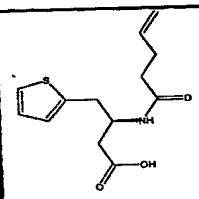
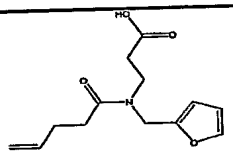
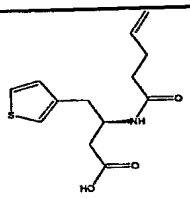
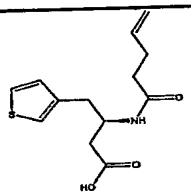
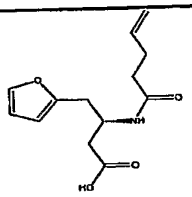
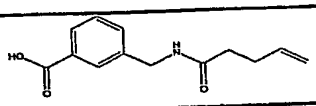
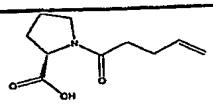
Oligo A22	ATCAC	BB_A22	
Oligo A23	TATCC	BB_A23	
Oligo A24	GGAAG	BB_A24	
Oligo A25	CGGTC	BB_A25	
Oligo A26	TGCTT	BB_A26	
Oligo A27	TTAGC	BB_A27	
Oligo A28	GCTGA	BB_A28	
Oligo A29	GAACG	BB_A29	
Oligo A30	CATGG	BB_A30	
Oligo A31	TGGTA	BB_A31	

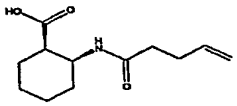
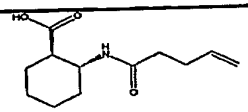
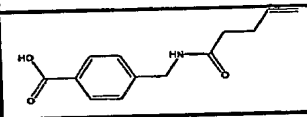
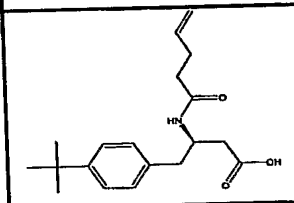
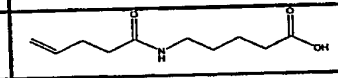
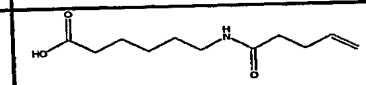
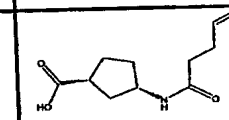
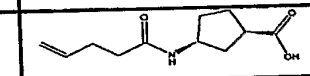
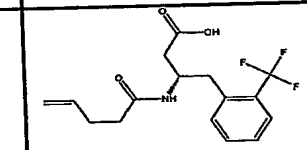
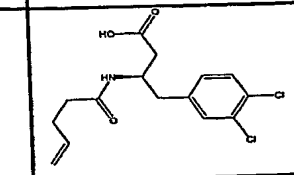
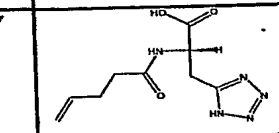
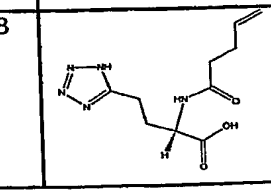
Oligo A32	TCAAG	BB_A32	
Oligo A33	ATCGA	BB_A33	
Oligo A34	ATGCA	BB_A34	
Oligo A35	ACTAG	BB_A35	
Oligo A36	TACCT	BB_A36	
Oligo A37	TACGA	BB_A37	
Oligo A38	CTTCA	BB_A38	
Oligo A39	CTCTT	BB_A39	
Oligo A40	TCATC	BB_A40	

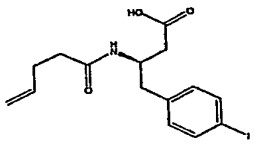
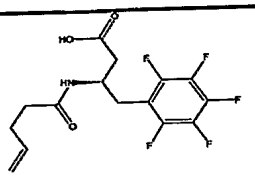
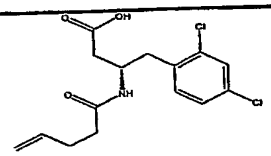
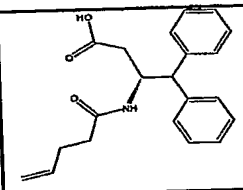
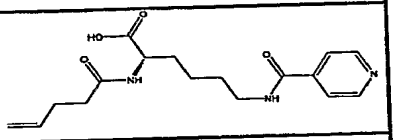
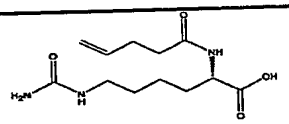
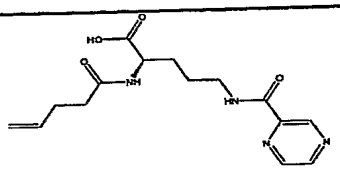
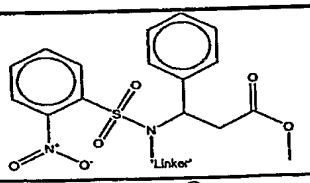
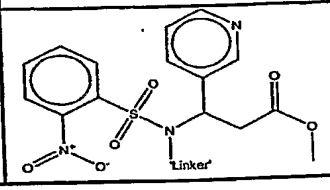
Oligo A41	ATTCC	BB_A41	
Oligo A42	CGACG	BB_A42	
Oligo A43	CCTGT	BB_A43	
Oligo A44	CCTTC	BB_A44	
Oligo A45	ACACC	BB_A45	
Oligo A46	TAACA	BB_A46	
Oligo A47	TAACA	BB_A47	
Oligo A48	CCAGG	BB_A48	
Oligo A49	ATGTC	BB_A49	

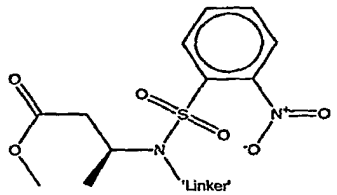
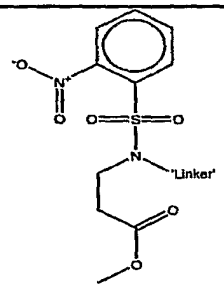
Oligo A50	GAGGA	BB_A50	
Oligo A51	GGTCA	BB_A51	
Oligo A52	GACTT	BB_A52	
Oligo A53	GGTGG	BB_A53	
Oligo A54	CAACT	BB_A54	
Oligo A55	ATGAG	BB_A55	
Oligo A56	TCTGC	BB_A56	
Oligo A57	ATAGG	BB_A57	
Oligo A58	CTACC	BB_A58	

Oligo A59	AAGTG	BB_A59	
Oligo A60	TCCAA	BB_A60	
Oligo A61	GCTCT	BB_A61	
Oligo A62	GGAGT	BB_A62	
Oligo A63	AATCG	BB_A63	
Oligo A64	AAGCT	BB_A64	
Oligo A65	CCGAA	BB_A65	
Oligo A66	TTTGT	BB_A66	
Oligo A67	CCGTG	BB_A67	

Oligo A68	TTTCG	BB_A68	
Oligo A69	TGAGG	BB_A69	
Oligo A70	GTTGC	BB_A70	
Oligo A71	AACTA	BB_A71	
Oligo A72	AACTA	BB_A72	
Oligo A73	CCTCG	BB_A73	
Oligo A74	AGCAA	BB_A74	
Oligo A75	TTCCA	BB_A75	
Oligo A76	AGACT	BB_A76	

Oligo A77	AGGTT	BB_A77	
Oligo A78	GCGTC	BB_A78	
Oligo A79	AACGT	BB_A79	
Oligo A80	CAAGA	BB_A80	
Oligo A81	AGAGA	BB_A81	
Oligo A82	GTACT	BB_A82	
Oligo A83	TAGAG	BB_A83	
Oligo A84	ACGAT	BB_A84	
Oligo A85	GACCA	BB_A85	
Oligo A86	TCGTT	BB_A86	
Oligo A87	GTCTC	BB_A87	
Oligo A88	CAGCA	BB_A88	

Oligo A89	TAGTC	BB_A89	
Oligo A90	GGGTG	BB_A90	
Oligo A91	CTCAG	BB_A91	
Oligo A92	AGAAC	BB_A92	
Oligo A93	GCGAG	BB_A93	
Oligo A94	GATGT	BB_A94	
Oligo A95	TCACT	BB_A95	
Oligo A96	CGTCT	BB_A96	
Oligo A97	AGCTC	BB_A97	

Oligo A98	CACTC	BB_A98	
Oligo A99	CAGTT	BB_A99	

Second round of library generation (round B) :

25 different combinations of BB_Bx and [oligo Bx-oligo bx] pairs were used.

Onto the free NH_2-group of the above loaded and deprotected BB_Ax building blocks were loaded the below listed BB_Bx building blocks:

Oligo Bx general structure :

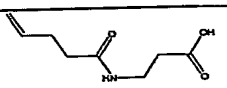
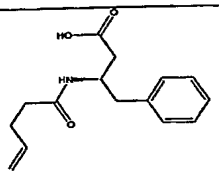
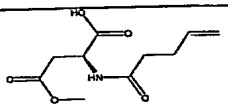
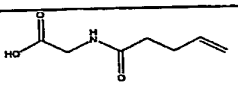
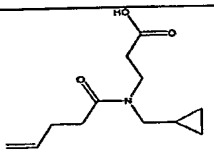
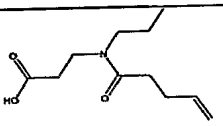
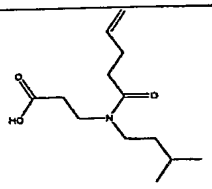
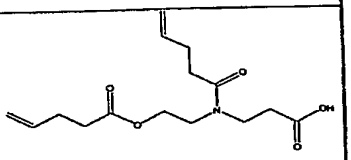
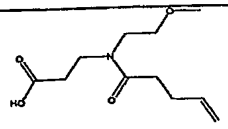
5'- **HPO_3**-CACAAGTACGAACGTGCATCAGAG-3'

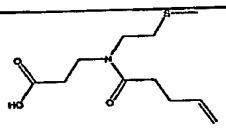
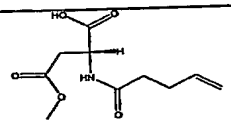
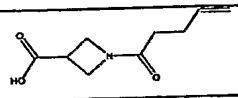
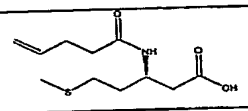
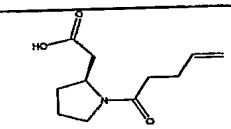
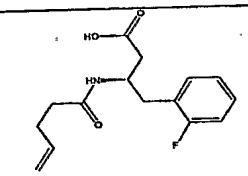
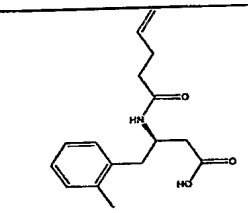
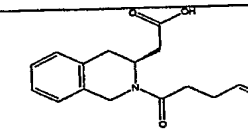
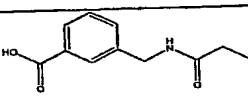
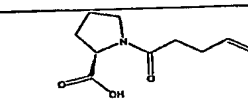
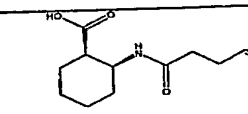
The underlined part corresponds to the 20 nucleotide sequence that varies among different B oligos, ie. the codon. The remaining sequence is identical among the B oligos.

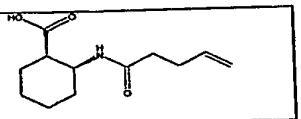
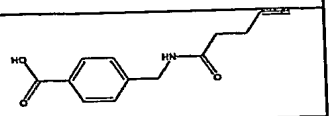
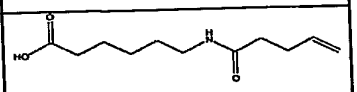
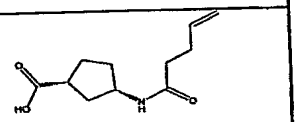
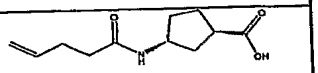
The corresponding b oligos used have the general structure :

5'-HPO_3- TCCTCTCTGATGCACGTTCGTACT

Every b oligo can anneal to a specific B oligo. As can be seen the above shown B oligo can anneal to the above shown b oligo.

Oligo	Codon	Building block	Building block structure
Oligo B1	AGTACGAACGTGCATCAGAG	BB_B1	
Oligo B2	TAGTCTCCTCCACTTCCATG	BB_B2	
Oligo B3	TACATCGTTCCAGACTACCG	BB_B3	
Oligo B4	TCCAGTGCAAGACTGAACAG	BB_B4	
Oligo B5	AGCATCACTACTCTGTCTGG	BB_B5	
Oligo B6	TCTTGTCAACCTTCCATGCG	BB_B6	
Oligo B7	AAGGACGTTCCTAGTAGGTG	BB_B7	
Oligo B8	GGAACCATCAAGATCCTGAG	BB_B8	
Oligo B9	ATCTCTGACGAGATCCAAGG	BB_B9	

Oligo B10	TCAAGGTTGGTGGTGTACTG	BB_B10	
Oligo B11	TCGAACTTGTTGCTTCCTCG	BB_B11	
Oligo B12	CTGAGTGTGTAGTACCAACG	BB_B12	
Oligo B13	ATCTTGGTTGTTCTCCTGCG	BB_B13	
Oligo B14	TAGTAGCTTGGAGTAGACCG	BB_B14	
Oligo B15	TTCACTCCATGCAGCATGTG	BB_B15	
Oligo B16	ACGATGGTGATCGATCAACG	BB_B16	
Oligo B17	TTCAGTGCTTGAGCTACCTG	BB_B17	
Oligo B18	TTGGACTCTTCTTGCACCAG	BB_B18	
Oligo B19	TCAACCAACTGGTTCTTGGG	BB_B19	
Oligo B20	TAGTACTCTACACTGCTGCG	BB_B20	

Oligo B21	TACACCATGACTTGCAGACG	BB_B21	
Oligo B22	GCATCTTGAGTCGTTGAACG	BB_B22	
Oligo B23	GACTCATCTCACTGGAGTTG	BB_B23	
Oligo B24	TCCAGCTTCTAGGAAGACAG	BB_B24	
Oligo B25	CTTCTTGAGTGCACTAGCAG	BB_B25	

Third round of library generation (round C) :

25 different C oligos were used :

Oligo Cx general structure :

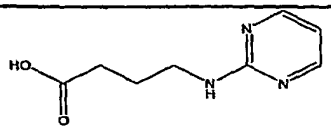
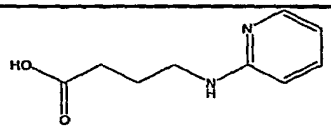
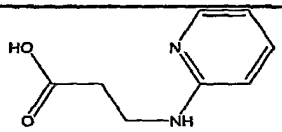
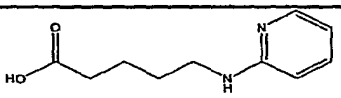
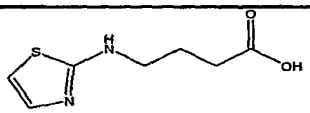
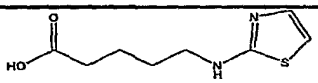
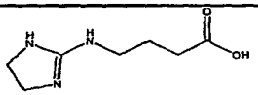
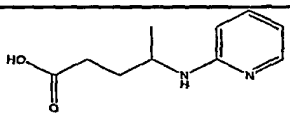
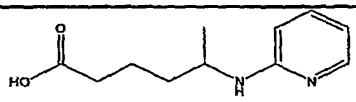
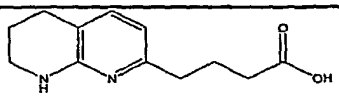
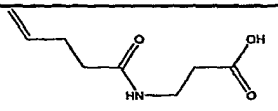
5'- HPO_3-CACAAGTACGAACGTGCATCAGAG-3'

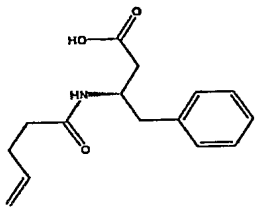
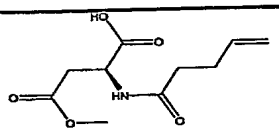
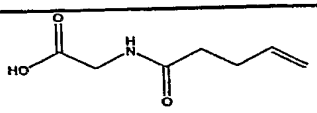
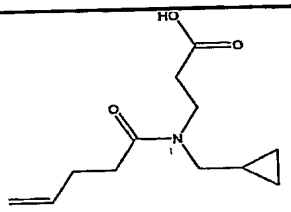
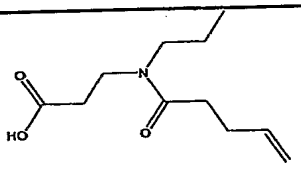
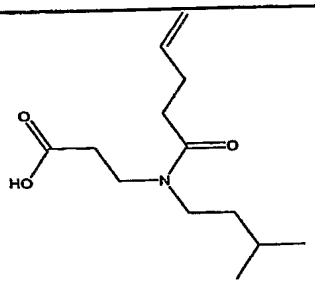
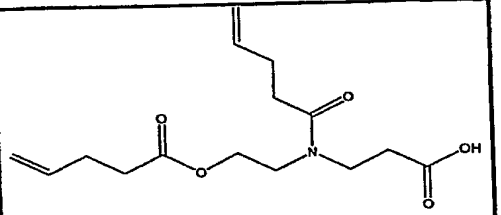
The underlined part corresponds to the 20 nucleotide sequence that varies among different C oligos, ie. the codon. The remaining sequence is identical among the C oligos.

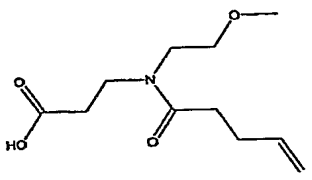
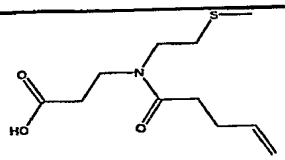
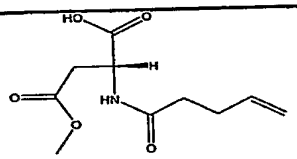
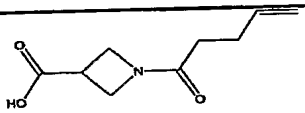
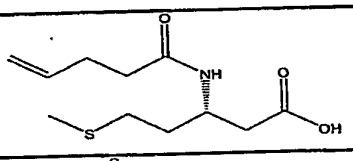
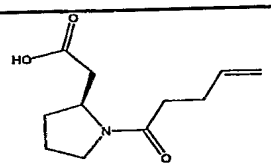
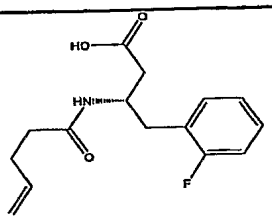
Onto these oligos were loaded the listed BB_Cx building blocks:

Oligo Cx example : 5'-HPO_3-
AGGACGAGCAGGACCTGGAACCTGGTGCGTTCCTCCACCACGTCTCCG

Oligo cx example : 5'- GCACCAGGTTCCAGGTCCTGCTCG

Oligo	Codon	Building block	Building block structure
Oligo C1	CGAGCAGGACCTGGAACCTGGTGC	BB_C1	
Oligo C2	CTCGACCACTGCAGGTGGAGCTCC	BB_C2	
Oligo C3	CGTGCTTCCTCTGCTGCACCACCG	BB_C3	
Oligo C4	CCTGGTGTCGAGGTGAGCAGCAGC	BB_C4	
Oligo C5	CTCGACGAGGTCCATCCTGGTCGC	BB_C5	
Oligo C6	CGTGAGGAGCAGGTCCTCCTGTCG	BB_C6	
Oligo C7	CCTGACACTGGTCGTGGTCGAGGC	BB_C7	
Oligo C8	CCATCTCGACGACCTGCTCCTGGG	BB_C8	
Oligo C9	CCACGAGGTCTCCACTGGTCCAGG	BB_C9	
Oligo C10	CCACTGAGCTGCTCCTCCAGGTGG	BB_C10	
Oligo C11	CCTCCTGTCCTGCACGTCCATCCG	BB_C11	

Oligo C12	CAGCACCTGGAGGTAGGACCACGG	BB_C12	
Oligo C13	CGACCAGACGAGGACCAGGTAGGC	BB_C13	
Oligo C14	CCAGGTTCGAGGACCTCGTCAGCC	BB_C14	
Oligo C15	CGAGCACGAGGAGCACGTGTCCAG	BB_C15	
Oligo C16	CCACGTCCACAGGTGCACCAGGTG	BB_C16	
Oligo C17	CCTGGTGCTCCACGACGTGCTTCG	BB_C17	
Oligo C18	CACGTGACGACCTGGTCAGGTGGG	BB_C18	

Oligo C19	CGTAGCTCGTGCTGGTCCTCCTGG	BB_C19	
Oligo C20	CGACGACCACCACCTTGGACACCC	BB_C20	
Oligo C21	CCTACGTCGTGCTCACGTCCTGCC	BB_C21	
Oligo C22	CGACGACAGCTAGGAGGAGGTGGG	BB_C22	
Oligo C23	CTGGTGGAGCTGCACGAGCACAGC	BB_C23	
Oligo C24	CAGGACTGGACGACGACCAGGTCG	BB_C24	
Oligo C25	CGATGCTGCAGACGACCAGCACCC	BB_C25	

Final extension

Oligo d: 5'-CGGAGACGTGGTGGAGGAAC

5

Results of sequencing the identifier oligonucleotides that result from the target selection

Fraction of templates encoding any binder before selection (estimated):1 in 61.875

Sequenced identifier oligonucleotides after selection (codons corresponding to small molecule X are shown in bold italics):

» G052.abd (53)
CGGCAGCCATGGCGTCGCACAATCTTGGTTGTTCTCCTGCGAGGACCATCTCGAACGACCTGGGGGTT
» F012.abd (53)
CGGCAGCACGATCGTCGCACAGGAACCATCAAGATCCTGAGGGGACCTGACACTGGTCGTGGTCGAGGCGTT
» E02.abd (53)
CGGCAGCAACTACGTCGCACATAGTCTCCTCCACTTCCATGAGGACCATCTCGACGACCTACTCCTGGGGTT
» E032.abd (52)
CGGGTGCCAAGGTGGTGGTCGTCGTCTCGCAGGAGAACAACCAAGATTGTGCGACGGCTAAGCTGCCGCT
» E03.abd (53)
CGGCAGCACACCCGTCGCACATTCACTCCATGCAGCATGCGAGGACCTGACACTGGTCGTGGTCGAGGCGTT
» H022.abd (52)
CGGCAGCGTACTCGTCGCACATCAAGGTTGGTGGTGTAACTGAGGACTCGACCACTGCAGGTGGAGTTCCGTT
» E01.abd (53)
CGGCAGCTTTGTCGTCGCACATCGAACTTGTTGCTTCCTCGAGGACCATCTCGACGACCTGCTCCTGGGGTT
» H042.abd (52)
CGGCAGCCTTACCGTCGCACATCCAGTGCAAGACTGAACAGAGGACCTGGTTGTCGAGGTGAGCAGCAGCGTT
» F042.abd (53)
CGGCAGCCTTACCGTCGCACATCAACAACTGGTTCTTGGGAGGACCACGTCCACAGGTGCACCAGGTGGTT
» E05.abd (53)
CGGCAGCCCTCGCGTCGCACATAGTCTCCTCCACTTCCATGAGGACCTGGTGCGCCACGACGTGCTTCGGTT
» E04.abd (52)
CGGCAGCGTTATCGTCGCACATCCAGTGCAAGACTGAACAGAGGACCACTGAGCTGCTCCTCCAGGTGGGTT
» G032.abd (53)
CGGCAGCTAACACGTCGCACATCCAGCTTCTAGGAAGACAGAGGACCTACCTCGTGCTCACGTCCTGCCGTT
» F062.abd (52)
CGGGTGCTGGTCGTCTGCAGCATCGTCCTCTGCTAGTGCACTCAAGAAGTGTGCGACGGGAATGCTGCCGCT
» H062.abd (53)
CGGCAGCGGATACGTCGCACAAGTACGAACGTGCATCAGAGAGGTCGACCCTGCAGGTGGAGCTCCGTT
» G062.abd (54)
CGGCAGCAGCTCCGTCGCACATCCAGTGCAAGACTGAACAGAGGACAGCACCTGGAGGTAGGACCACGGGTT
» E022.abd (53)
CGGCAGCTAACACGTCGCACATCGAACTTGTTGCTTCCTCGAAGGACCACTGAGCTGCTCCTCCAGGTGGGTT
» E012.abd (53)
CGGGTGCTGGTCGTCTGCAGCATCGTCCTCTGCTAGTGCACTCAAGAAGTGTGCGACGGGAATGCTGCCGCT
» H012.abd (53)
CGGCAGC*ATTCC*CGTCGCACA*CTTCTTGAGTGCACTAGCAG*AGGA*CGATGCTGCAGACGACCAGCACCC*GTT

» E062.abd (53)
CGCGACCAGGATGGACCTCGTCGAGTCCTCTGTTCAGTCTTGCACTGGATGCGACGGCACTGCTGCCGCT
» F032.abd (53)
GGAACCTGGACAGTTGGAGACCTCGTGGTCCTCGTCTACAAGTCATGGTGTATGTGCGACGACCAGGCTGCCGCT
» F022.abd (52)
CGGCAGCCGCACCGTCGCACAGCATCTTGAGTCGTTGAACGAGGACTCGACCACTGCAGGTGGAGCTCCGTT
» H052.abd (53)
CGGCAGCACACCCGTCGCACAGGAACATCAAAGATCCTGAGAGGACCATCTCGACGACCTGCTCCTGGGGTT
» G022.abd (53)
CGGCTGACGAGGTCCTCGAACTGGTCCTCACCTAGTAGGAACGTCCTTTGTGCGACGAGTTGGCTGCCGCT
» G042.abd (53)
CGGCAGCTCTGCCGTCGCACACTTCTTGAGTGCACTAGCAGAGGACCACGAGGTCTCCACTGGTCCAGGTT
» E042.abd (55)
CGCTGCTGCTCACCTCGACACCAGGTCCTCCTTGGATCTCGTCAGAGATTGTGCGACGGCTCGGCTGCCGCT
» E052.abd (54)
CGGCAGCCACTCCGTCGCACACTGAGTGTGTAGTACCAACGAGGACGAGCACGAGGAGCACGTGTCCAGCGTT
» G012.abd (54)
CGGCAGCGTTCCGTCGCACATTCAGTGCTTGAGCTAACTGAGGACACTCGTNGATGATCCTGCTACCGTNGGGTT
» H032.abd (53)
CGGCAGCATCCTCGTCGCACATAGTAGCTTGGTACGTATGACCGAGGACCACAGAAGGTCTCCACGTGGTCCAGGTT

Fraction of identifiers encoding binder X after selection before MISE treatment: 1 out of 28. Enrichment fold in selection : (1/27)/(1/61.875) = 2210 fold (theoretical maximum is 61875 fold).

<u>Results of sequencing the identifier oligonucleotides that result from mismatch selection (MISE) (codons corresponding to small molecule X are shown in bold italics):</u>

» MISE5_r140 (69) GGCAGC***ATTCCC***GGTCGCACA***CTTCTTGAGTGCA***–
CTAGCAGAGGA***CGATGCTGCAGACGACCATGCACCCG***TTC
« MISE5_r112 (192) GGCAGC***ATTCCC***GTCGCACA***CTTCTTGAGTGCA***–
CTAGCAGAGGA***CGATGCTGCAGACGACCAGCACCCG***TTC
« MISE5_r117 (183) GGCAGC***TATTCC***ACTGTCGCTACA***CTTCTTGTAGTGCA***–
CTAGCAGAGGA***CGATGCTGCATACAGACCAGCACCCG***TTC
« MISE5_r115 (201)
GGCAGC***ATTCTC***TCGTCGCACA***CTTCTTGAGTGCAGCTAGCAG***AGGAT***CGATGCTGCATGACGATCCAGCACCCG***T
« MISE5_r111 (194)
GCTAGCA***TTCG***CCGTTGCACA***CTTCTTGAGTGCAGTAGCAG***AGGA***CGATGCTGCAGACGAGCCAGCACCCG***TTC
« MISE5_r148 (232)
GGCAGC***ATTCCC***GTCGCACA***CTTCTTGAGTGCATTAGCAG***AGGA***CGATGCTGCAGAGCGACCAGCACCCG***TTC
« MISE5_r123 (208)
GGCAGCGTTTCGCGTGCGCACA***CTTCGTTGAGTGCAATCTAGCAG***AGGACTGATGCTGCTAGACGACCAGCACCCGTT

```
< MISE5_r137  (212)
GGCAGCATTCCCGTCGCACACTTCTTGAGTGCAGTAGCAGAGGACGATGCTGCANACGAGCCAGCACCCGTTC
< MISE5_r144  (228)
GGCAGCATTCCACGTCGCTACACTTCTTGAGTGCACTAGTCAGAGGANGATGCTGCANACGACCCAGCACCCGTTC
< MISE5_r132  (215)
TGGCAGCATTCCCGTCGCACACTTNNTGAGTGCACTAGCATGAGGATCGATGCTGCAGAGCTACCAGCACCCGTT
< MISE5_r126  (235)
GGCAGCATGTCCCGTCGCTACGCTTCTTGAGTGCATCTAGGCAGAGGACGATGGGCTGCAGACGACCAGCACCCGTT
< MISE5_r105  (247)
GGCAGCATTCCCGTCGCACACTTTCTTGAGTGCAACTAGCAGAGGACGATGTCTGCAGACGACCAGCACCCGTTC
< MISE5_r131  (298)
GGCAGCATTCCCGTCGCACACTTCTTGAGTGCACTAGCAGAGGACGATGCTGTGCAGACGACCAGCACCCGTT
```

Fraction of templates encoding binder X after MISE treatment : 12 out of 13
Enrichment fold : (12/13)/(1/28) = 26 (theoretical maximum is 28 fold).

The output of the selection process shows 26 different sequences. Thus, it is not possible to rank the corresponding display molecule in accordance with their affinity towards the integrin target. The subsequent Mismatch Selection allows the clear conclusion that a single display molecule prevails over all others.

Example 9

A library composed of 10^7 different identifier oligonucleotides of 165 units were assembled according to the general methods of example 8. This library was PCR amplified. The amplicons was denaturated at 95 degree Celsius and allowed to rehybridisere for 2 hours at room temperature. The rehybridised product were run on a standard 4% agarose gel next to a specific identifier oligonucleotide at room temperature. The result of the experiment is shown in Fig. 5, in which lane 1 shows the DNA size marker, lane 2 shows the 10^7 identifier oligonucleotide library, and lane 3 shows a specific identifier oligonucleotide.

2A: DNA smear corresponding to heteroduplexes in the 10^7 member library
2B: Band corresponding to homoduplexes in the 10^7 member library

3A: Distinct band corresponding to homoduplex of specific identifier oligonucleotide.

Example 10

The same identifier oligonucleotides as produced in example 9 were used.

Lane 1: Distinct band corresponding to homoduplex
Lane 2: 10^7 identifier oligonucleotide library
Lane 3: DNA size marker
Lane 4: 10^7 identifier oligonucleotide library
Lane 5: 10^7 identifier oligonucleotide library, heat-denatured

1A: Distinct band corresponding to homoduplex
2A: DNA smear corresponding to heteroduplexes in the 10^7 member library
2B: Distinct band corresponding to homoduplexes in the 10^7 member library
4A: DNA smear corresponding to heteroduplexes in the 10^7 member library
4B: Distinct band corresponding to homoduplexes in the 10^7 member library
5A: DNA smear corresponding to heteroduplexes in the 10^7 member library

As can be seen in lanes 4 and 5, the homoduplexes formed in the PCR amplified 10^7 member library (band 4B) are denatured by heat (no band corresponding to homoduplexes in lane 5) resulting in only heteroduplexes being present. A period of renaturation following PCR amplification allows homoduplex formation (lanes 2 & 4).

As can be seen, homoduplexes can be resolved on the gels enabling the isolation and PCR reamplification of these. Thus this is an iterative method for enriching homoduplexes.

Claims

1. A method for obtaining display molecule(s) having affinity towards a target, comprising the steps of
   a. providing a library comprising a plurality of different display molecules, each display molecule being associated with an identifier oligonucleotide, which codes for the identity of said display molecule,
   b. contacting the library with a target to allow for an interaction between the display molecules of the library with the target,
   c. partitioning a fraction enriched in identifier oligonucleotides of display molecules interacting with the target,
   d. subjecting the fraction to denaturing conditions and subsequently to conditions at which homo-duplexes renaturate,
   e. recovering the homo-duplexes, and
   f. deducing from the homo-duplexes the identity of the display molecule(s) interacting with the target.

2. The method according to claim 1, wherein in step c the identifier oligonucleotides of the library members are provided in homo-duplex form.

3. The method according to any of the claims 1 and 2, wherein in step d, the renaturing conditions favours formation of homo-duplexes, while formation of hetero-duplexes is avoided.

4. The method according to any of the claims 1 to 3, wherein the renaturing conditions include that a mixture of hetero-duplexes and homo-duplexes is formed.

5. The method according to any of the claims 1 to 4, wherein the homo-duplexes in step e are recovered by removal of hetero-duplexes and single stranded identifier oligonucleotides.

6. The method according to claim 5, wherein the hetero-duplexes are removed by enzymatically degradation.

7. The method according or claim 6, wherein the enzyme is a nuclease.

8. The method according to any of the claims 5 to 7, wherein the enzyme is selected from T4 endonuclease VII, T4 endonuclease I, CEL I, nuclease S1, or variants thereof.

9. The method according to any of the claims 6 to 8, wherein the enzyme is thermostable.

10. The method of claim 1, wherein the display molecule is a reaction product of two or more chemical entities and the identifier oligonucleotide comprises codons identifying the chemical entities.

11. The method according to claim 10, wherein the chemical entities are precursors for a structural unit appearing in the display molecule.

12. The method according to any of the claims 10 to 11, wherein some or all of the chemical entities are not naturally occurring α-amino acids or precursors thereof.

13. The method according to claim 10, wherein each codon comprises 4 or more nucleotides.

14. The method according to any of the claims 1 to 13, wherein the display molecules of the library are non-α-polypeptides.

15. The method according to any of the claims 1 to 14, wherein the display molecules of the library are non-nucleic acids.

16. The method according to any of the claims 1 to 15, wherein the display molecule has a molecular weight less than 2000 Dalton, preferably less than 1000, and most preferred less than 500 Dalton.

17. The method according to any of the preceding claims, wherein the identifier oligonucleotide uniquely identifies the display molecule.

18. The method according to any of the claims 1 to 17, wherein one or more chemical entities are transferred to the nascent display molecule by a chemical building block further comprising an anti-codon.

19. The method according to claim 18, wherein the information of the anti-codon is transferred in conjunction with the chemical entity to the nascent complex.

20. The method according to any of the preceding claims, wherein the chemical entities are reacted without enzymatic interaction.

21. The method according to any of the claims 1 to 20, wherein the codons are separated by a framing sequence.

22. The method according to any of the claims 1 to 21, wherein a selectively cleavable linker joins the display molecule and the identifier oligonucleotide.

23. The method according to claim 22, wherein the linker is cleaved by irradiation.

24. The method according to any of the claims, wherein the library comprises one, two or more different library members.

25. The method according to any of the claims 1 to 24, wherein the library comprises 1,000 or more different members.

26. The method according to claim 1, wherein the molecular target is of a biological origin.

27. The method according to any of the claims 1 to 26, wherein the molecular target is immobilized on a solid support.

28. The method according to claim 27, wherein the target immobilized on the support forms a stable or quasi-stable dispersion.

29. The method according to claims 27 or 28, wherein a cleavable linker is present between the solid support and the molecular target.

30. The method according to any of the claims 1 to 29, wherein the molecular target is a protein.

31. The method according to claim 30, wherein the protein is selected from the group consisting of kinases, proteases, phosphatases, and anti-bodies.

32. The method according to any of the claims 1 to 30, wherein the molecular target and/or the display molecule is a nucleic acid.

33. The method according to claim 32, wherein the nucleic acid is a DNA or RNA aptamer.

34. The method according to any of the claims 30 to 33, wherein the target protein is attached to the nucleic acid responsible for the formation thereof.

35. The method according to any of the claims 1 to 34, wherein the contacting step includes that a target is mixed with a library of complexes.

36. The method according to claim 35, wherein a target is saturated with a known ligand prior to the mixing step.

37. The method according to claim 1, wherein the recovered homo-duplexes of step e are amplified prior to decoding the identity of the display molecule.

38. The method according to any of the claims 1 to 37, wherein the partitioned fraction of identifier oligonucleotides of step c is amplified by PCR prior to step d.

39. The method of claim 38, wherein the identifier oligonucleotides are proportionally amplified.

40. The method according to any of the claims 1 to 39, wherein the recovered homo-duplexes of step e one or more times are recycled to step d.

41. The method according to claim 40, wherein the recovered homo-duplexes are amplified prior to the treatment according to step d.

42. The method according to claims 39 or 40, wherein a decoding occurs before recycling to step d.

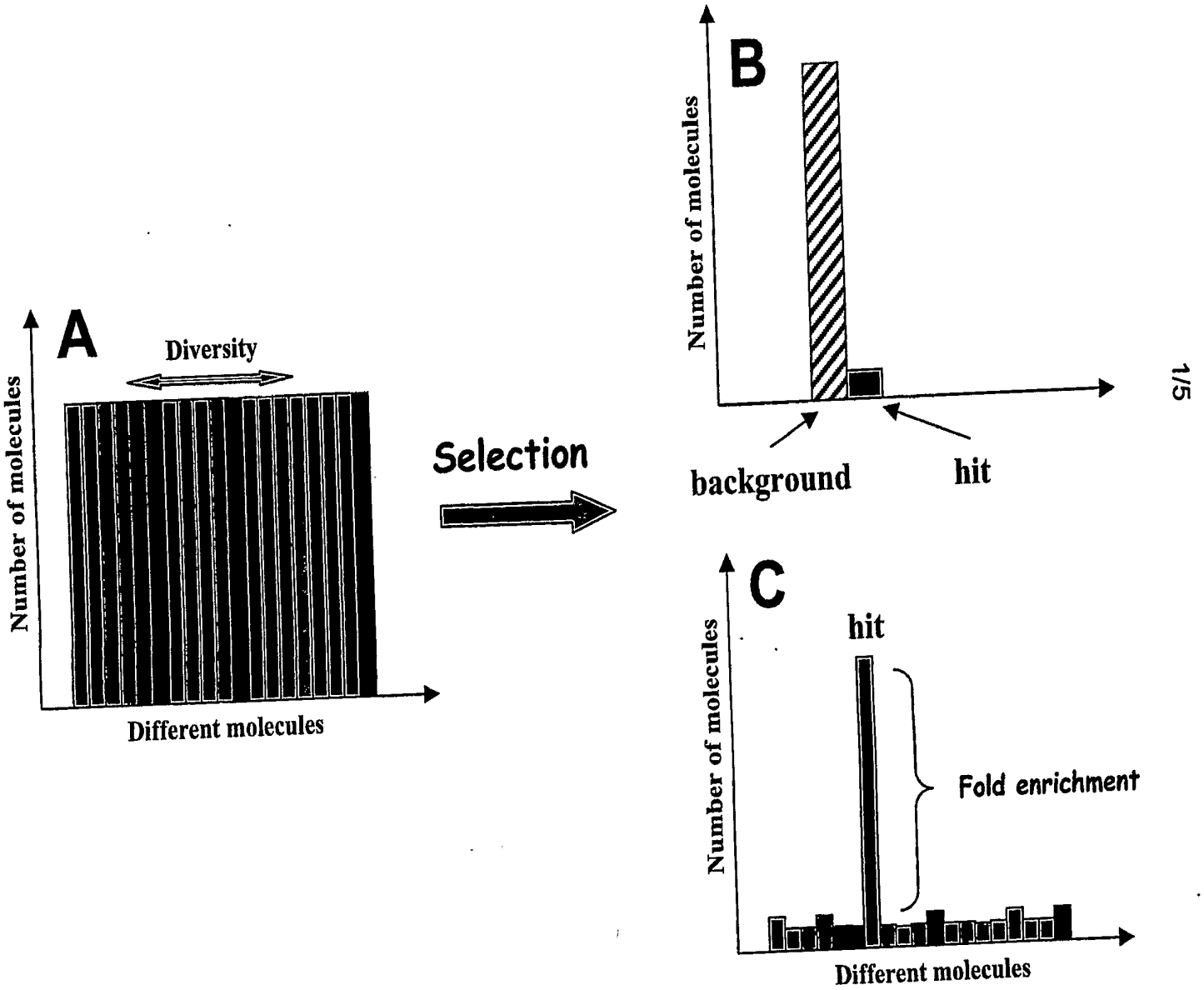
43. The method according to any of the claims 40 to 42, wherein the information obtained from the decoding is used to modify the composition of the identifier oligonucleotides or complements thereof before recycling to step d.

44. The method according to claim 42, wherein the modification includes extinction of certain identifiers.

Abstract

The present invention relates to a method for selecting a molecule from a library of such molecules associated with identifier oligonucleotides, said molecule having affinity towards a target. The method involves contacting the library with a target to allow for an interaction between the molecules and the target and partitioning a fraction enriched in identifier oligonucleotides of molecules interacting with the target. After an optional nucleic acid amplification of the partitioned fraction, the fraction is subjected to denaturing conditions and subsequently to renaturing conditions at which homo-duplexes are formed. The homo-duplexes are subsequently recovered and decoded to identify the identity of the molecule interacting with the target.

Fig. 1.



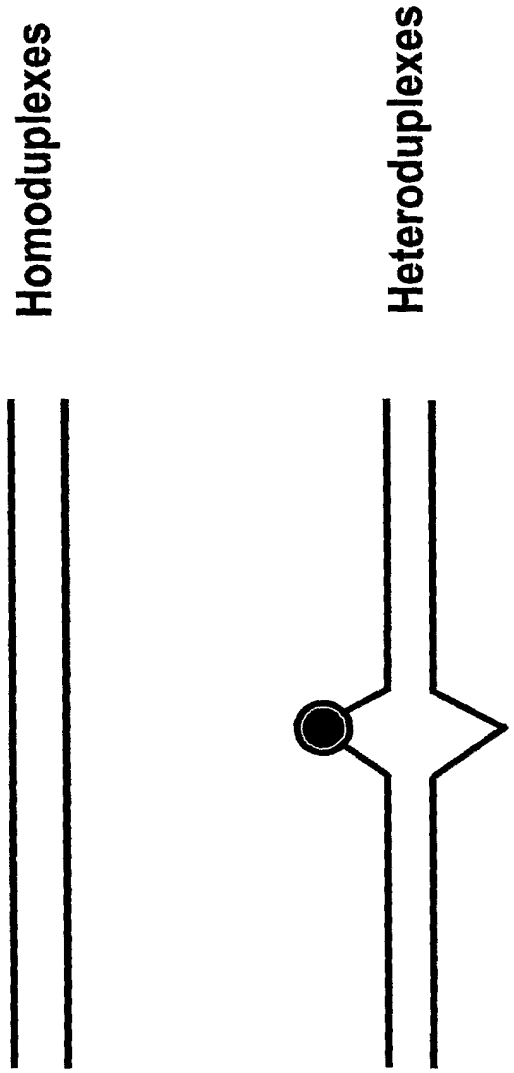


Fig. 2

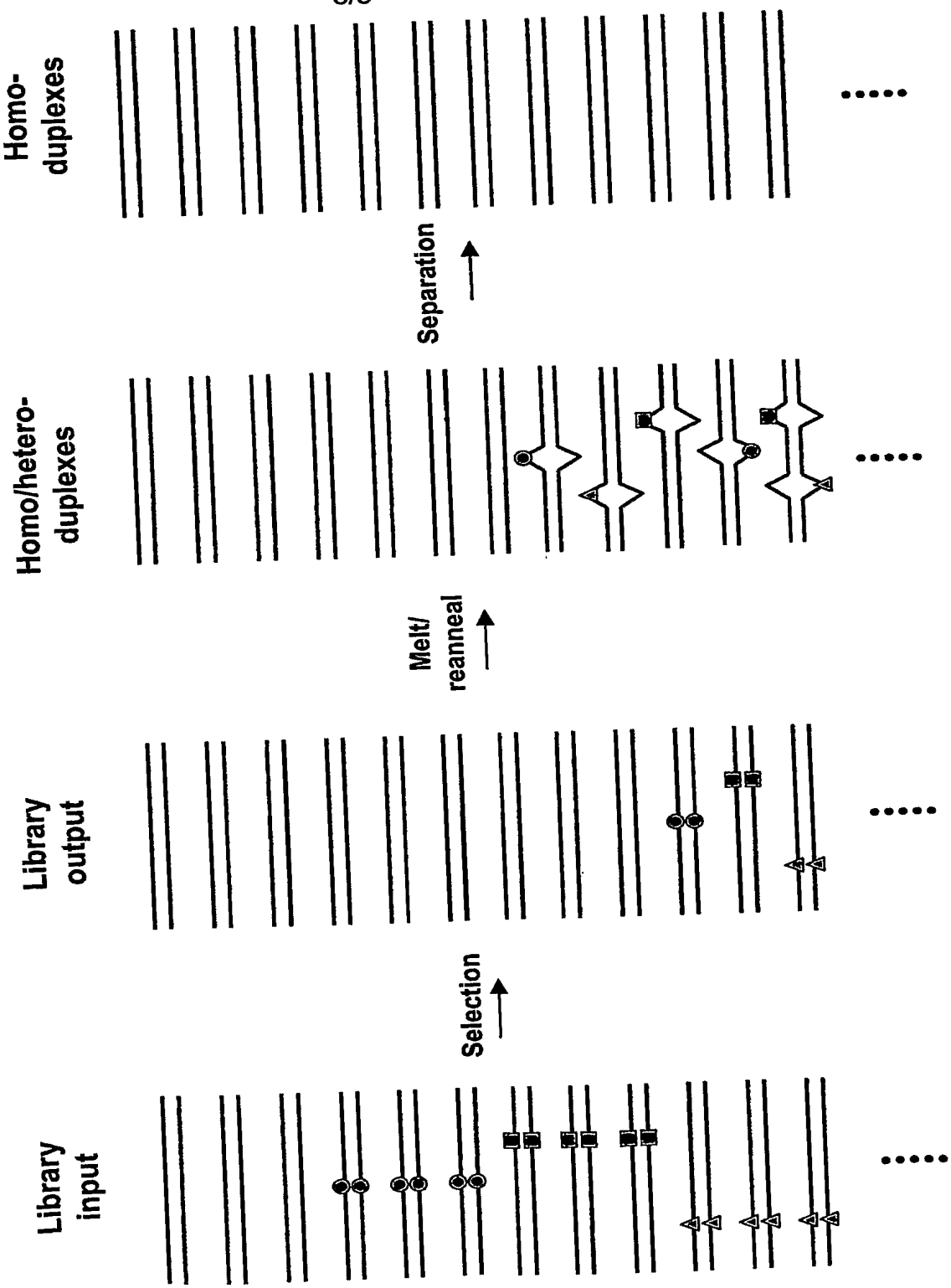
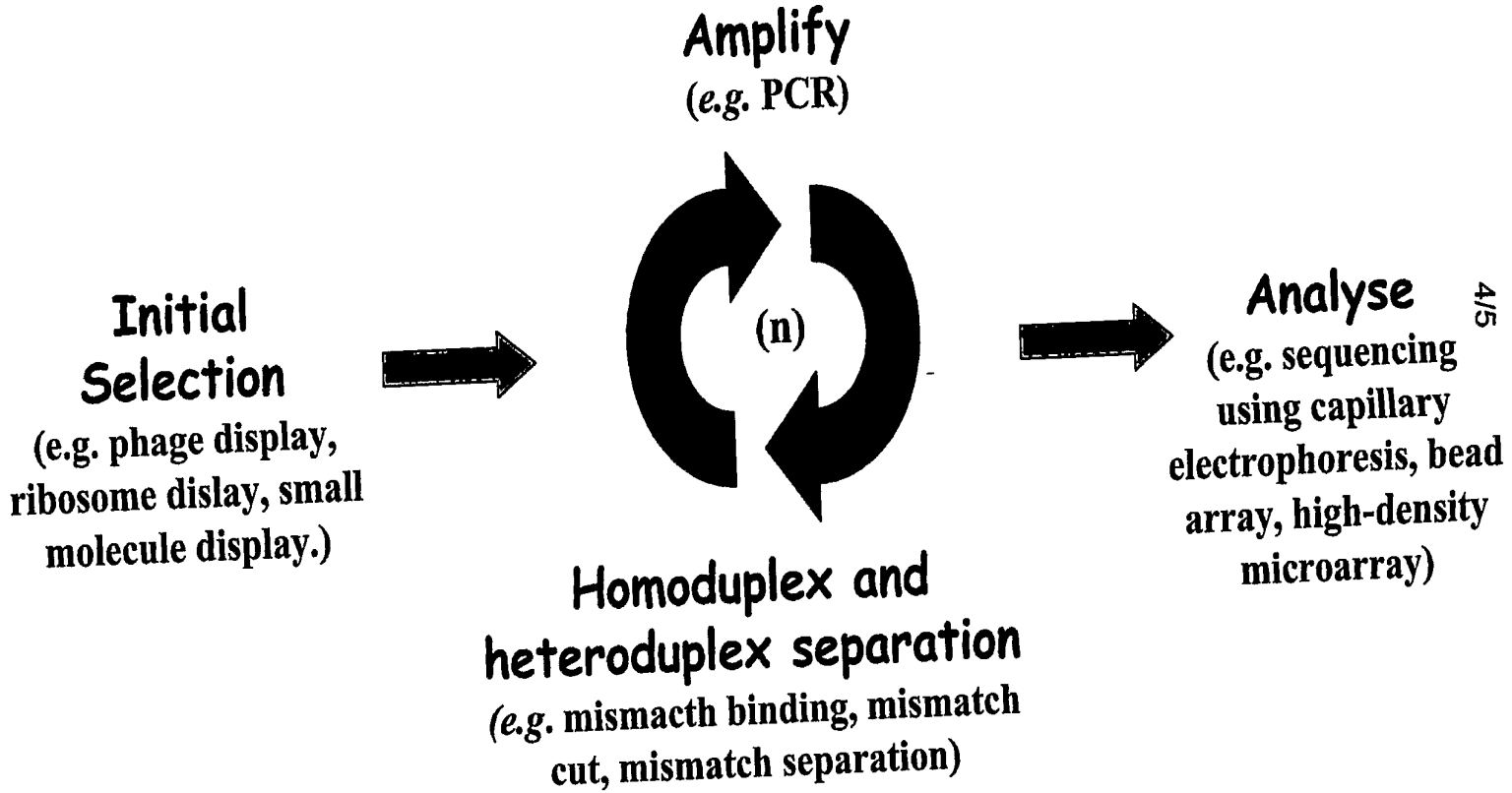
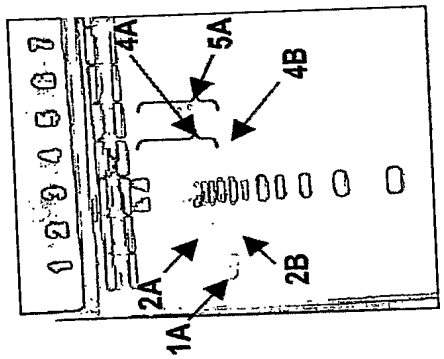


Fig. 3

Fig. 4



Panel B



Panel A

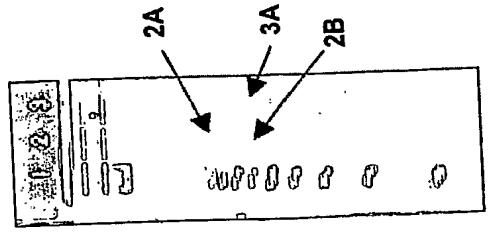


Fig. 5